# IDENTIFICATION AND CHARACTERIZATION OF A NEW GENE ESSENTIAL FOR PRODUCTION OF FORMATE HYDROGENLYASE ACTIVITY IN *ESCHERICHIA COLI*

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

# IDENTIFICATION AND CHARACTERIZATION OF A NEW GENE ESSENTIAL FOR PRODUCTION OF FORMATE HYDROGENLYASE ACTIVITY IN ESCHERICHIA COLI

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A hitherto unidentified gene (fhlC) essential for the synthesis and activity of formate hydrogenlyase (FHL) in Escherichia coli was identified by isolating a lac-fusion strain defective in FHL activity. This mutant strain lacks both formate dehydrogenase (BV- and PMS-linked) activities and a hydrogenase activity associated with the FHL system. The defect in the fhlC mutant strain was overcome by addition of either 1 uM molybdate to glucose minimal medium or 100 uM molybdate to LB/glucose medium. These results

suggest that the gene defective in the mutant strain is involved in molybdenum transport in *E. coli*.

The *fhlC* gene was mapped at approximately 66 minutes in the *E. coli* chromosome. The wild type *fhlC* gene was cloned in a recombinant plasmid and found to reside within a 2.86 kb EcoRI-KpnI fragment of *E. coli* chromosomal DNA insert present in this plasmid. The physical mapping of the *fhlC* gene by transposon Tn5 mutagenesis showed that the DNA coding for the *fhlC* gene resides closer to the KpnI cleavage site than to the EcoRV site in the chromosomal DNA insert. The transcriptional orientation of the *fhlC* gene was found to be clockwise along the *E. coli* chromosome. The wild type $fhlC^{+}$ gene was dominant to the *fhlC* mutational allele.

The *fhlC* gene was expressed constitutively under both aerobic and anaerobic conditions as determined by the beta-galactosidase expression in this *lac*-fusion strain. The expression of the *fhlC* gene was independent of molybdate, nitrate, cAMP, formate and *fnr* gene product.

A defect in the *fhlC* gene abolished expression of three different genes (*fdhF*, *ant*, and *hyd*) essential for production of formate hydrogenlyase complex. The pleiotropic effects of the *fhlC* mutation on these genes were completely suppressed by addition of either molybdate or tungstate to the growth medium.

# INTRODUCTION

Production of hydrogen gas by the members of Enterobacteriaceae has been known since the turn of this century (76). During fermentative growth of these heterotrophic microorganisms, the energy requirement for cell growth is satisfied by degradation of a relatively large quantity of organic compounds. Under these conditions, the organic compounds are incompletely metabolized to produce various fermentative end products, most of which are organic acids. As a consequence of accumulation of organic acids, the pH of the culture medium decreases to a level below the optimum for growth of the organisms. In order to achieve higher cell yields, these bacteria tend to alleviate the decline in pH by further conversion of organic acids to neutral compounds. Escherichia coli, which is not known to produce neutral compounds (besides ethanol), oxidizes formate, generated as a fermentation product, to $H_2$ and $CO_2$. In many facultative, anaerobic, enteric bacteria, like E. coli, formate hydrogenlyase activity is induced under fermentative growth conditions and this enzyme complex converts formate to carbon dioxide and hydrogen gas (106). By providing an efficient way of decomposing formate, formate hydrogenlyase

contributes to pH stabilization during the fermentative growth of the organism (21). Thus, formate hydrogenlyase plays an important role in anaerobic metabolism and ultimately enhances the growth of fermentatively growing E. coli culture.

In spite of its importance in anaerobic metabolism, the physiological, biochemical and genetical mechanisms of regulation of the formate hydrogenlyase system are not well understood. Recently, a number of genes essential for the activity of this pathway have been reported (42, 62, 84, 89, 110). However, the regulation of these genes is poorly understood. In order to understand the regulation of this pathway, I have isolated a mutant strain of E. coli as a lambda placMu operon fusion strain which showed pleiotropic defects in components of formate hydrogenlyase pathway. In this study, the biochemical and genetic characteristics of this mutant strain and the role of the altered gene on the regulation of formate hydrogenlyase are presented.

## LITERATURE REVIEW

Fermentative production of $H_2$ and $CO_2$ from formate is catalyzed by a membrane-bound multienzyme complex, formate hydrogenlyase (FHL) (3). This FHL complex is a part of a complex interconnected system for anaerobic electron transport which also includes the formate/$NO_3$ pathway and $H_2$/fumarate pathway (48). The FHL complex consists of a formate dehydrogenase, hydrogenase, and unidentified electron carriers between these two enzymes (37, 85). The reaction catalyzed by this FHL enzyme complex is a non-energy yielding reaction operating at a $G_o'$ of Ø ($H_2/2H^+$, $E_o'$= -420mV; formate/$CO_2$ + $2H^+$, $E_o'$ = -420mV) (3).

In E. coli, two formate dehydrogenase (FDH) activities have been distinguished. One FDH activity is associated with nitrate-dependent anaerobic respiratory pathway (FDH1) and the other is associated with formate hydrogenlyase (FDH2) (41, 48). These two FDH activities can be distinguished by their ability to donate electrons to artificial electron acceptors at different redox potentials. The FDH1 preferentially reduces methylene blue (MB, $E_o'$ = + 11mV) or phenazine methosulfate (PMS, $E_o'$ = - 80mV) but not benzyl viologen (BV, $E_o'$ = -360mV) (85, 88). The FDH2 has the opposite specificity (85, 88). Using this substrate

specificity, the levels of the two FDH activities in the cell can be independently determined. These activities show differences in their induction patterns also. Both formate dehydrogenase activities are induced under fermentative growth conditions, when the FHL system is fully active. However, the FDH1 activity is maximally induced in the presence of nitrate (63, 85, 87). There are, however, a number of common properties shared by these two enzyme activities: the synthesis and activities of both FDH1 and FDH2 are induced by anaerobiosis and repressed in the presence of oxygen (38, 95, 106). Both enzymes require selenium and molybdenum for their activity (63, 86).

The FDH1 has been purified and characterized (31). It consists of three different subunits; 110 kd, 32 kd, 20 kd polypeptides. Selenium is associated with the 110 kd peptide. The enzyme also contains molybdenum. Although the FDH2 enzyme has not been purified, Cox et al. (23) reported that this enzyme possesses an 80 kd selenopeptide. This protein is different from the 110 kd selenopeptide present in FDH1. Partially purified FDH2 differs from FDH1 in their kinetic properties, substrate (electron acceptors) specificities and subcellular localization (87) as well as immunological characteristics (36). These results suggest that the two enzymes are structurally different although they may share some common component.

A gene, mapping at 80 min in the *E. coli* chromosome, has been implicated to be the structural gene coding for the

110 kd selenopeptide of FDH1 (48). Bock and his coworkers (84) isolated a Mud1(Ap, lac) insertion mutant defective in FDH2 activity. These investigators mapped the mutation (fdhF) at 92.4 minutes in E. coli chromosome. This fdhF gene was shown to be the structural gene for the 80 kd selenopeptide of FDH2 (83). These results demonstrate that the two FDH activities are coded by two different genes. However, it is not clear whether the two proteins share any structural components.

Hydrogenase is involved in the final step of the FHL system. Electrons generated from formate oxidation, by FDH2, are transferred to hydrogenase and converted to hydrogen gas using protons as electron acceptor. E. coli is also capable of utilizing $H_2$ as a reductant for growth under anaerobic conditions, if an appropriate electron acceptor, like fumarate, is present in the medium ($H_2$ uptake) (69). Under these growth conditions, hydrogenase catalyzes the oxidation of $H_2$ gas and the electrons so generated are transferred through an electron transport chain to fumarate reductase which catalyzes the reduction of fumarate to succinate. The oxidation of $H_2$, coupled to fumarate reduction, leads to the production of 1 mole of ATP/mole of fumarate reduced (55). This energy-conserving anaerobic respiratory pathway is called hydrogen uptake (HUP) system. This HUP system enables E. coli to use hydrogen as an energy source. Therefore, in E. coli, hydrogenase has two distinctive activities associated with two different

anaerobically inducible metabolic pathways: $H_2$ evolution activity (reduction of protons) associated with the FHL system, and $H_2$ uptake activity (oxidation of $H_2$) linked to the HUP system. The question that arises is whether the two hydrogenase activities are catalyzed by the same enzyme or by two different enzymes.

Several investigators have reported that the crude extracts of E. coli contain multiple hydrogenases, when assayed for activity with viologen dyes as electron acceptors, after fractionation in native polyacrylamide gels (1, 109). These observations raised the possibility that E. coli produces multiple hydrogenases. However, Adams and Hall (2) purified a single hydrogenase from aerobically/microaerobically grown E. coli cells. The apparent molecular weight of this protein was reported to be 113 kd and this protein contained two identical subunits of 56 kd. Using anaerobically grown cells, Patel (81) also isolated a single hydrogenase with similar biochemical properties to those of the hydrogenase purified by Adams and Hall (2). Recently, Boxer and his coworkers demonstrated immunologically distinct multiple hydrogenase isoenzymes in the membrane fraction of anaerobically grown E. coli (9, 91). Two of these enzymes, termed hydrogenase 1 and 2, were purified and characterized. Isoenzyme 1 contained two polypeptide subunits of 64 kd and 35 kd, although its physiological function was not clearly defined (91, 92). An active tryptic fragment of isoenzyme 2 was reported to have

180 kd apparent molecular weight and to be composed of equimolar amounts of subunits of 61 kd and 30 kd (10). This enzyme was suggested to be the hydrogenase component involved in hydrogen uptake pathway (HUP-hydrogenase) (9, 91). These investigators showed that neither hydrogenase 1 nor hydrogenase 2 was part of formate hydrogenlyase and proposed the presence of a third isoenzyme for FHL-hydrogenase activity (91). The relationships between these isoenzymes and previously isolated hydrogenase are not clearly established.

Regulation of the formate hydrogenlyase pathway in E. coli has been reported to be different from that of the interconnected electron transport pathway: formate/$NO_3$ and $H_2$/fumarate pathway. The FHL activity is not detectable in aerobically grown cultures (106). The FHL activity can be induced upon transfer of an aerobic culture to anaerobic conditions. However, the presence of nitrate or fumarate, terminal electron acceptors which induce formate/$NO_3$ pathway and $H_2$/fumarate pathway, respectively, suppresses FHL activity (28). These experimental results suggest that anaerobiosis and absence of terminal electron acceptors (oxidants) are two main requirements for expression of FHL activity. The presence of formate in the medium has been shown to enhance the FHL activity (22). However, it is not known whether formate hydrogenlyase is produced in the absence of endogenous formate production. Low pH of the growth medium, a condition generally encountered by E. coli

growing anaerobically, was also reported to enhance the FHL activity (38).

The activity and synthesis of FDH2 were also reported to be controlled by the same physiological factors as those of the FHL system. It is also induced by anaerobiosis, repressed by aerobic conditions, and by nitrate (6, 28, 106). The activity of FDH2 was also enhanced by the presence of formate in the medium (84). This activity was also increased by low pH in the medium (38).

E. coli hydrogenase is also regulated by several control systems. Hydrogenase activity is not detectable in aerobically grown E. coli (41, 48). Anaerobically grown cells exhibited hydrogenase activity, while the hydrogenase activity was absent in cells grown in the presence of nitrate (58, 84, 107). Since there are two hydrogenase activities in E. coli cells (FHL-hydrogenase activity and HUP-hydrogenase activity), these experimental results suggest that both hydrogenase activities are repressed by the presence of these electron acceptors ($O_2$, $NO_3^-$).

Addition of formate to an anaerobic culture has been reported to enhance the levels of hydrogenase activity in the cell (84). Krasna (58) reported that the hydrogenase activity is higher in cells grown in $H_2$/fumarate medium than in glucose minimal medium. Lee et al. (62) also reported that the hydrogenase activity is enhanced by the addition of formate or $H_2$, the substrates for FHL and HUP system, respectively, in a chlA mutant strain which is defective in

both FDH activities. These results indicate that formate and $H_2$ act as inducers for hydrogenase synthesis in E. coli.

These earlier studies on the regulation of hydrogenase synthesis in E. coli imply that the hydrogenase activities are regulated by different physiological factors. Recently, Sawer et al. (91) analyzed the cellular contents of hydrogenase isoenzymes by cross immunoelectrophoresis by use of the antibody prepared against hydrogenase 1 and 2. Levels of hydrogenase 2 (HUP-hydrogenase) was specifically enhanced after growth with either hydrogen and fumarate or glycerol and fumarate, and correlated with cellular hydrogen uptake activity. However, in the presence of formate in medium, cellular contents of neither of these isoenzymes were correlated with formate hydrogenlyase activity, although an increase in total hydrogenase activity could be observed. These investigators attributed the increase in total hydrogenase activity to an unidentified hydrogenase isoenzyme 3 (FHL-hydrogenase). These results showed that the two hydrogenase activities (FHL-hydrogenase and HUP-hydrogenase) were catalyzed by two different hydrogenase isoenzymes, although the role of hydrogenase 1 in the cell was unclear. Thus, in order to understand the regulation of hydrogenase synthesis and activity, it is essential to distinguish among these hydrogenase activities. The regulatory elements should be assessed for their separate effect(s) on these hydrogenase activities.

A number of genes which are essential, for production of formate hydrogenlyase enzyme activity, were also reported. Since the FHL system consists of formate dehydrogenase 2, hydrogenase and unidentified electron carriers, mutation in any of the genes essential for the production of each of the components in the pathway would also abolish formate hydrogenlyase activity.

Several genes have been described to be essential for production of active formate dehydrogenase 2. These include fdhA (80 min), fdhB (38 min), fdhC (82 min), fdhD (83 min) and fdhF (92.4 min) (42, 83). The fdhF gene is known to be the structural gene of FDH2, but the functions of the other genes were not identified.

Some of the chlorate-resistant mutant strains (chl mutants) have been reported to be deficient in formate hydrogenlyase activity and formate dehydrogenase 2 activity (19, 34, 94, 102). Since the formate dehydrogenase 2 is a component of formate hydrogenlyase, the lack of formate hydrogenlyase activity in these mutant strains can be partly explained as a consequence of the absence of formate dehydrogenase 2 activity. These mutations were mapped at four different genetic loci in E. coli chromosome and designated as chlA (18 min), chlB (86 min), chlD (17 min) and chlE (18 min). These four chlorate mutant strains are pleiotropic in that they not only lack formate hydrogenlyase activity but also lack all molybdoenzyme activities in E. coli: nitrate reductase (43, 48), formate dehydrogenase 1

(48, 65), tertiary amine N-oxide reductase (101) and biotin-D-sulfoxide reductase (25). Because molybdenum is required for formate dehydrogenase 2 activity and these pleiotropic chl mutants lack formate dehydrogenase 2 activity, the formate dehydrogenase 2 enzyme might share a common molybdo-cofactor with other molybdoenzymes mentioned above. The common molybdo-cofactor has been identified and named molybdopterin (MPT) which is a complex of a novel 6-alkyl pterin with Mo atom (53, 54, 55). Strains with a mutation in chlB are postulated to lack a factor necessary for insertion of the Mo cofactor into the pterin (33, 68), and the chlA and chlE mutants are reported to be defective in the pterin biosynthesis. The chlD mutants are phenotyphically reversed to the wild type when 1 mM molybdenum was included in the medium (35). The observation that high concentration of molybdenum in the medium causing a phenotyphic reversal of the mutation to the wild type indicates that the defect is in Mo transport or processing rather than the MPT synthesis. Although properties of these mutant strains were well characterized in the nitrate reductase system, the effect of these mutations on formate hydrogenlyase is very poorly understood.

Three mutational alleles defective in electron carriers in the formate hydrogenlyase pathway were identified. These include ant (110), fhlA (90) and fhlB (unpublished data). Mutants carrying a mutation in ant and fhlA are deficient in FHL activity but produced decreased

level of FDH2 activity. However, fhlB mutant strain produced normal level of FDH2 activity but lacked FHL activity. Although the strains carrying a mutation in either one of these genes produced hydrogenase activity, the nature of the hydrogenase activity (FHL-hydrogenase or HUP-hydrogenase, or both) was not examined.

A number of genes essential for production of hydrogenase activity were also identified. These include hydA (62), hydB (62), hydC (108), hydD (108), hydE (105), hydF (89) and hydG (unpublished data). Mutations in these genes abolished total hydrogenase activity, suggesting that these genes are required for both FHL-hydrogenase and HUP-hydrogenase activity.

A pleiotropic anaerobic regulatory gene (fnr) which regulates the synthesis of fumarate reductase and nitrate reductase has also been implicated to affect the expression of hydrogenase and formate dehydrogenase 2 (60). Mutant strains with a mutation in the fnr gene produced a low level of hydrogenase activity (17% of parent level) and formate dehydrogenase 2 activity (25% of wild type level). Sawer et al. (91) demonstrated that the active fnr gene product is required for the full induction of hydrogenase 2 (HUP-hydrogenase) but not for hydrogenase 3 (FHL-hydrogenase). Thus, the role of fnr gene product on FHL activity is uncertain.

In summary, the physiological and biochemical data on the formate hydrogenlyase system show that the components of

the FHL system are different from those of the interconnected anaerobic electron transport pathways and that the regulatory mechanisms controlling the FHL system are also quite different from those of the anaerobic electron transport pathways. Although there are a number of genes identified as essential for an active FHL pathway, the role of these genes, with respect to the regulation of this unique metabolic pathway, is very poorly understood. In order to understand the regulation of the formate hydrogenlyase pathway, I have isolated a mutant strain defective in formate hydrogenlyase activity. Biochemical and genetic characteristics of this mutant strain and the role of the gene product on the regulation of formate hydrogenlyase production are presented in this dissertation.

## MATERIALS AND METHODS

### Strains

The genotype of the bacterial and phage strains used in this study and their pedigrees and sources are presented in Table-1.

### Materials

Biochemicals were purchased from Sigma Chemical Co. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were analytical grade. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc., Enzo Biochem. Inc., Boehringer Mannheim Biochemicals, or New England Bio Lab, Inc.

### Media

Basal minimal medium had the following composition: $Na_2HPO_4$, 6.25 g; $KH_2PO_4$, Ø.75 g; NaCl, 2.ØØ g; $(NH_4)_2SO_4$, 1.Ø g; $MgSO_4$ $7H_2O$, Ø.2 g; $FeSO_4$ $7H_2O$, Ø.Ø1 g; $NaMoO_4$ $2H_2O$, Ø.Ø1 g; $Na_2SeO_3$ $5H_2O$, Ø.263 mg in 1 liter of deionized water. The final pH of the medium was 7.5. For glucose minimal medium, the basal minimal medium was supplemented

Table 1. Strains used in this study

| Strain | Genotype or phenotype | Reference |
|---|---|---|
| *E. coli* | | |
| AT2446 | Hfr, thi-1, metC69, relA1, lambda-minus spoT1 | (103) |
| AT2699 | F⁻, hisG1, thyA3, metC69, argG6, lacY1, gal-6, malA1, rpsL, tsx-1, lambda-r, lambda-minus | (103) |
| BW545 | Δ(lacU)169, rpsL | (110) |
| CSH26 | F⁻, ara Δ(lac-pro), thi | (74) |
| GW5305 | BW545, ant4::Mu d1 (Ap, lac) | (110) |
| GW5310 | GW5305, lambda P1(209) lysogen | (110) |
| Hfr3000 | Hfr, thi-1, relA1, spoT1, lambda-minus, supQ80 | (7) |
| JC10244 | F⁻, cysC-43, alaS3, srl-300::Tn10, thr-1, leu-6, thi-1, lacY-1, galK2, ara-14, xyl-5, mtl-1, proA2, his-4, argE-3, rpsL-31, tsx-33, supE-44, lambda-minus | (62) |
| JRG861a | F⁻, gal, trpA9761, iclR, trpR, rpsL fnr | (60) |
| LCB898 | F⁻, thr-1, leu-6, tonA21, rpsL, lacY1, supE44, pfl-1 | (104) |
| LE392 | F⁻, supF, supE, hsdR, galK, trpR, metB, lacY, tonA | (85) |
| LS853 | F⁻, trpA9605, his-85, cya-2, trpR55, lambda-minus, IN(rrnD-rrnE)1 | (16) |
| M3s | MC4100, hyd::Mu d(Ap, lac) | (84) |
| M9s | MC4100, fdhF::Mu d(Ap, lac) | (84) |

Table 1 - continued.

| Strain | Genotype or phenotype | Reference |
|---|---|---|
| MBM7014 | $F^-$, araC(am), araD, Δ(argF-lac)U169, trp(am), malB(am), rpsL, relA, thi, supF | (11) |
| MC4100 | $F^-$, araD139, Δ(argF-lacU169), rpsL150, relA1, flbB5301, deoC1, ptsF25, rbsR | (18) |
| P3478 | $F^-$, thyA36, polA1, deoC2, lambda-minus IN(rrnD-rrnE)1 | (26, 44) |
| SE29 | Puig 426, hupA | (62) |
| SE31 | JC10244, hupB | (62) |
| SE42 | JC10244, hupB, $alaS^+$ | (62) |
| SE1001 | JC10244, $alaS^+$ | |
| SE1100 | BW545, fhlC::lambda plac Mu | |
| SE1101 | BW545, fhl::lambda plac Mu | |
| SE1102 | BW545, fhl::lambda plac Mu | |
| SE1103 | BW545, fhl::lambda plac Mu | |
| SE1119 | Hfr3000, fhlC::lambda plac Mu | |
| SE1130 | SE1100, $fhlC^+$, zgg-3::Tn10 | |
| SE1142 | JRG861a, $fnr^+$, zcj-5::Tn10 | |
| SE1147 | LS853, $cya^+$, zif-4::Tn10 | |
| SE1157 | MC4100, icd, zgg-3::Tn10 | |
| SE1158 | MC4100, icd, zgg-3::Tn10 | |
| SE1162 | LS853, cya, zif-4::Tn10 | |
| SE1187 | SE1100, lambda p1(209) | |

Table 1 - continued.

| Strain | Genotype or phenotype | Reference |
|---|---|---|
| SE1188 | JRG861a, *fnr*, *zcj*-5::Tn10 | |
| SE1194 | SE1100, *cya*, *zif*-4::Tn10 | |
| SE1212 | SE29, *hupA*$^{+}$, *zgf*-1::Tn10 | |
| SE1226 | SE1100, *fnr*, *zcj*-5::Tn10 | |
| SE1237 | LCB898, *pfl*$^{+}$, *zba*-6::Tn10 | |
| SE1261 | SE29, *hupA*, *zgf*-1::Tn10 | |
| SE1267 | LCB898, *pfl*, *zba*-6::Tn10 | |
| SE1308 | SE1100, *pfl*, *zba*-6::Tn10 | |
| SE1319 | P3478, *fhlC*::Tn5 | |
| SE1325 | SE1001, *fhlC*::Tn5 | |
| SE1328 | M3s, *fhlC*::Tn5 | |
| SE1334 | M9s, *fhlC*::Tn5 | |
| SE1433 | SE1100, *icd*, *zgg*-3::Tn10 | |
| SE1438 | CSH26, *fhlC*::lambda *plac* Mu | |
| SE1443 | SE1438/F'ts114 zzf::Tn10 | |
| SE1448 | SE1433/F'122 | |
| SE1449 | SE1100, *hupA*, *zgf*-1::Tn10 | |
| SE1450 | SE1449/F'116 | |
| SE1455 | SE1100, *hupA*, *zgf*-1::Tn10 | |
| SE1459 | SE1443, Hfr | |
| SE1473 | GW5310, *fhlC*::Tn5 | |

Table 1 - continued.

| Strain | Genotype or phenotype | Reference |
|---|---|---|
| *S. typhimurium* | | |
| TT627 | *str*A1, *pyr*C7/F'ts114 *zzf*::Tn10 | (20) |
| Phages | | |
| P1 | Tn9, $Cm^r$, *c1r*-100 | (74) |
| lambda NK421 | *b*221, *rex*::Tn5, *c*I857, *O*am23, *P*am80 | (85) |
| lambda NK561 | *b*221, *c*I::Tn10, *O*am29, *P*am80 | (32) |
| lambda p1(209) | (*b*-*xis*)[(+'Mu)::(*trp*'BA'*lac*'OZY)] | (18) |
| lambda p*lac*Mu 53 | *imm*, '*trp*'*lacZ*$^+$*lacY*$^+$*lacA*'$_t$ '*uvrD*', *xho*::*kan*, Mu[*c*$I_{ts}$62,*ner*$^+$*A*$^+$*S*] | (15) |
| lambda pMu 507 | *c*I857, *S*am7, Mu[*c*$I_{ts}$62, *ner*$^+$*A*$^+$*B*$^+$] | (15) |

with glucose to a final concentration of 0.3% for aerobic growth and 1.5% for anaerobic growth. Glycerol/fumarate medium was prepared by addition of glycerol (1.5%) and fumarate (40 mM) to the basal minimal medium. Hydrogen/fumarate (HF) medium was used to test the ability of E. coli cells to grow under anaerobic conditions, utilizing hydrogen as electron donor and fumarate as electron acceptor. The composition of HF medium was described previously (2). Luria broth (LB) was prepared as described previously (74). Glucose and sodium formate were added to the LB at a final concentration of 0.3% and 0.1%, respectively, whenever needed. When required, the growth medium was also supplemented with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 20 ug/ml), ampicillin (100 ug/ml), kanamycin (50 ug/ml), streptomycin (100 ug/ml) or tetracycline (20 ug/ml). Maltose/tetrazolium (2,3,5,-triphenyl tetrazolim chloride; TTC) and McConkey/lactose media were prepared as described previously (74, 97).

Phage P1 diluent contained 10 g/l bacto-tryptone and 10 mM $MgCl_2$. Phage P1 adsorption medium consisted of 5 mM $CaCl_2$ and 10 mM $MgCl_2$. Soft agar overlay (LCTG) for growing phage P1 contained $CaCl_2$ (2.5 mM), glucose (60 mM), agar (0.6%) and thymine (25 mg/l) in LB. Phage lambda diluent was 10 mM Tris-HCl (pH 7.5) containing $MgSO_4$ (10 mM), NaCl (50 mM) and gelatin (0.1%).

## Growth Conditions

Anaerobic bacterial growth on solid medium was achieved in an anaerobic jar containing GasPak (BBL, Microbiology Systems) or in a vaccum desiccator, under $N_2$ atmosphere, at room temperature. Growth was determined after 3-5 days. In order to test the ability of different strains to utilize $H_2$ as electorn donor and fumarate as electron acceptor for their growth, cultures were streaked onto HF-agar medium and incubated in the vacuum dessicator, under $H_2$ atmosphere. Growth was scored after 7 days of incubation, at room temperature.

Cells used for enzyme assays were grown in appropriate liquid medium, under anaerobic conditions. Aerobically grown, 16 h old, LB cultures were used as inoculum [5% (vol/vol)]. For preparation of cell-free extracts, cells were grown in 1 liter of medium. By filling the culture vessel to the top with the medium, the culture was maintained anaerobic. For determination of enzyme activities using whole cells, screw cap tubes (16 x 150 mm) were filled to the top and stoppered tightly after inoculation. Cultures grown in LB medium and glucose-minimal medium were harvested after 4 h and 6 h of incubation, respectively, at $37^{o}C$.

For beta-galactosidase assay, mutants carrying lac fusions were grown in 20 ml of liquid medium in 70 ml serum bottle sealed with serum stopper. The gas phase in the serum bottle was replaced with nitrogen. The inoculum size

and incubation period were the same as described above. For aerobic growth of these lac fusion strains, cells were grown in 10 ml of appropriate medium in 125 ml Erlenmeyer flask and incubated at 37°C, with shaking (200 rpm), for 2 h (LB media) or 3 h (glucose minimal medium). The inoculum size for these experiments were 5% (v/v) of 16 h old aerobically grown culture.

## Biochemical Assays

### Preparation of Whole Cells and Extracts for Enzyme Assay

For whole cell enzyme assys, cells from 20 ml culture were harvested by centifugation, at 3,500 x g, for 5 min at room temperature and washed once with 10 ml of the wash buffer [0.1M NaK-$PO_4$ buffer (pH 7.0) containing 1 mM reduced glutathione and 100 ug/ml of chloramphenicol]. The cells were resuspended in 1.5 ml of the same buffer. The cell suspensions were maintained in ice, under $N_2$.

For preparation of extracts, cells from a 1 liter culture were centrifuged at 8,000 x g, for 10 min, at 4°C. The cells were resuspended in 10 ml of wash buffer and centrifuged again at 12,000 x g (4°C), for 10 min. The cells were resuspended in 1 ml of wash buffer and passed through a French pressure cell, at 20,000 $lb/in^2$. This broken cell suspension was centrifuged at 20,000 x g, for 20 min, at 4°C and the supernatant was collected. The crude extract was maintained in ice, under an $N_2$ atmosphere.

Cell protein in whole bacterial cells was determined as described before (62). Under these experimental conditions, one optical density unit (measured at 420 nm in a Spectronic 710 spectrophotometer) corresponded to 350 ug cell protein per ml. Protein concentration of cell-free extracts was determined by previously described procedures (14, 29). Bovine serum albumin was used as the protein standard.

Hydrogenase and Hydrogen Uptake (HUP) Activities

Hydrogenase activity in whole cells was measured by two different assay methods. These include a tritium exchange assay (4, 64) and reduction of an artificial electron acceptor, benzyl viologen (BV), by hydrogenase using $H_2$ as the electron donor, a method used by other investigators (79). The tritium exchange reaction is independent of electron transport proteins (4) and provides an actual measure of the hydrogenase activity present in the cell.

For tritium exchange assay, a cell suspension containing approximately 50 ug of cell protein was placed in a 12 x 75 mm test tube. The sample volume was adjusted to 0.2 ml with the wash buffer. The assay tube was sealed with a serum stopper (11 x 17 mm). The gas phase was replaced with helium. Tritium gas (11.2 mCi/mmol; New England Nuclear Corp.) was added (25 ul) to a final concentration of 0.55 uCi per assay. After 1 hr of incubaction, at room temperature, the serum stopper was removed, and the tritium

gas was vented in the hood, for 10 min, after vigorous mixing of the tube contents. Tritiated water, present in a 50 ul fraction was determined with a scintillation counter after adding 2.5 ml of Scintiverse-E scintillation fluid. Hydrogenase activity was expressed as nanomoles of tritiated water produced, per hour, per milligram of cell protein. Production of tritiated water from $^3H_2$ was linear with time over the entire assay period and also was proportional to the concentration of cell protein (up to 100 ug cell protein).

Hydrogenase and hydrogen uptake activities were also determined by monitoring the disappearance of $H_2$ from the gas phase, by using a gas chromatograph, in the presence of either BV or fumarate, as electron acceptors. The assay mixture for these reactions contained NaK-$PO_4$ buffer (0.1 M; pH 7.0), BV or fumarate (50 mM) and a cell suspension at a final concentration of 150 to 200 ug of cell protein (in a final volume of 1 ml) in a 10-ml Wheaton vial (45). The vial was closed with a serum stopper and an aluminum seal and the gas phase was replaced with $N_2$. Hydrogen gas was added to a final concentration of 10%. The amount of $H_2$ present in the gas phase was determined at different time intervals, using a Varian gas chromatograph (model 920) with a molecular sieve 5-angstrom column. The enzyme activity was expressed as nanomoles of $H_2$ consumed, per min, per mg of cell protein.

Formate Dehydrogenase 1

The activity of formate dehydrogenase 1 (FDH1) was assayed spectrophotometrically as formate-dependent (phenazine methosulphate-mediated) reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue, MTT). The assay mixture contained, in a final volume of 3 ml, phosphate buffer (pH 6.5, Ø.33 M); sodium formate (4Ø mM); phenzine methosulfate (98 uM); MTT (6Ø mM) and cell extract or whole cells. The reaction was carried out at room temperature, in an anaerobic cuvette, under $N_2$ atmosphere. The enzyme activity was measured by monitoring the rate of MTT reduction, at 56Ø nm, using a Spectronic 71Ø spectrophotometer. If whole cells were used for assay, a SLM-Aminco-DW2C spectrophotometer was the instrument of choice. The amount of reduced formazan produced was calculated by using a molar extinction coefficient of $1.44 \times 1Ø^4$ $cm^{-1}$. The enzyme activity was expressed as nmol of reduced formazan produced, per min, per mg protein or cell protein.

Formate Dehydrogenase 2

Formate dependent BV reduction, a measure of formate dehydrogenase 2 (FDH2) was determined spectrophotometrically at 55Ø nm. The assay mixture contained phosphate buffer (pH 7.Ø, Ø.33 M); BV (6.5 mM); sodium formate (4Ø mM); cell extract or whole cells. The final volume was adjusted to

3.0 ml with deionized water. The reaction was carried out in an anaerobic cuvette, at room temperature, in an $N_2$ atmosphere. The amount of BV reduced was calculated by using a molar extinction coefficient of $7.78 \times 10^3$ $cm^{-1}$. The enzyme activity was expressed as nanomoles of reduced-BV produced, per min, per mg of protein or cell protein.

Fumarate Reductase

Fumarate reductase activity was assayed spectrophotometrically by measuring fumarate-dependent oxidation of reduced BV. The assay mixture contained phosphate buffer (pH 7.8, 92.5 mM); BV (350 uM); sodium fumarate (30 mM); and cell extract. The reaction volume was 20 ml (94). The reaction was carried out under argon atmosphere, in an anaerobic cuvette, at room temperature. The reaction was initiated, by addition of sodium dithionite to reduce BV (to an absorbance of 2.0 at 550 nm). The rate of oxidation of reduced BV was continuously monitored and converted to the amount of fumarate reduced by the enzyme (99). The enzyme activity was expressed as nmol of reduced-BV oxidized, per min, per mg protein.

Formate Hydrogenlyase

The activity of formate hydrogenlyase (FHL) was assayed by measuring the rate of $H_2$ production from formate. The reaction mixture contained phosphate buffer (pH 6.5, 0.33

M), sodium formate (40 mM) and cell extract or whole cells (45). The final volume was 1.0 ml. The reaction was carried out at room temperature, in a 10-ml Wheaton vial, under $N_2$ atmosphere. The rate of production of $H_2$ was monitored with a gas chromatography. The activity was expressed as nmol of $H_2$ produced, per min, per mg of protein or cell protein.

Beta-galactosidase

Assay procedure used for beta-galactosidase activity was as described by Miller (74). The cells used in the assay were permeabilized using chloroform and sodium dodecyl sulfate (SDS).

## Genetic Methods

Transduction

Transduction experiments were carried out as described by Miller (74) with few modifications, using bacteriophage P1 Cm clr100 (74).

Recipient cells were grown in LB medium to a density of about 5 x $10^8$ cfu/ml. The cells were sedimented by centrifugation, at room temperature and resuspended in 1.0 ml of P1 adsorption medium and infected with the phage at a multiplicity of infection (M.O.I.) of 1 to 5. The bacteria and phage mixture was incubated at room temperature, for 30 min, before sedimenting the cells by centrifugation to

remove uninfected phage particles. The cell pellet was resuspended in Ø.5 ml of LB or basal minimal medium. Transductants were selected on appropriate selective media, at 25°C. Phage P1-free transductants were selected after curing the cells by growing at 42°C.

Phage Preparation

Bacteriophage P1 Cm clr1ØØ was grown in an appropriate host strain using confluent plate lysis method. Cells from an overnight LB culture of the host strain were harvested and resuspended in the same volume of P1 adsorption medium. To Ø.1 ml of the cell suspension, $1Ø^5$-$1Ø^6$ phage particles were added. After 3Ø min of incubation at room temperature, for phage adsorption, 2.5 ml of LCTG-soft agar (5Ø°C) was added and the mixture was poured over freshly prepared LB-agar containing thymine (1ØØ ug/ml). The plates were incubated at 42°C until confluent lysis of cells occurred (usually 8-1Ø h). The top, soft agar layer was removed with 2.5 ml of P1 diluent and thoroughly mixed in the presence of several drops of $CH_3Cl$. The chloroform-treated extract was centrifuged at 1Ø,ØØØ x g, for 1Ø minutes, at 4°C. The supernatant was collected and stored, at 4°C, over several drops of chloroform. All the p1 lysates were titered using strain SE1ØØ1 in which the phage produces slightly larger plaques.

Lambda phages (lambda NK421,lambda NK 561, lambda placMu 53, and lambda pMu 5Ø7) were grown in strain LE392 by

plate lysis method. Strain LE392 was grown at 37°C, for 16 h, in LB containing 0.4% maltose. To 0.1 ml of the culture, $MgSO_4$ was added to a final concentration of 10 mM. Approximately $10^5$ lambda phage particles were added to the cell suspension. After 30 min incubation at room temperature, for phage adsorption, 2.5 ml of water-Thy agar containing 0.6% agar and thymine (100 ug/ml) was added to the tube contents and poured on freshly prepared LB agar. The plates were incubated at 37°C for about 8 h until confluent lysis of cells. Lambda diluent (2.5 ml) was added to the plate and the top soft agar layer was removed to a centrifuge tube. After mixing the soft agar with pippet, in the presence of several drops of $CH_3Cl$, the tube contents were centrifuged at 10,000 x g, for 10 min. The supernatant containing the phage particles was collected and stored, at 4°C, over several drops of chloroform. Strain LE392 was also used for titering the phage preparations.

Lambda phage P1(209) was prepared by UV induction of the phage from a lysogenic strain (GW5310). Strain GW5310 was grown to the early to mid-log phase of growth in LB (about 3 x $10^8$ cfu/ml). Cells were sedimented after centrifugation, at 3,500 x g, for 5 min, at room temperature, and resuspended in 1 ml of minimal medium. The cell suspension was irradiated with UV (500 uW/$cm^2$.sec) for 10 sec. The UV irradiated cells were resuspended in 10 ml of LB containing 10 mM $MgSO_4$ and incubated in a rotary shaker at 37°C for 5 h. After cell lysis was noticable, 0.5

ml of $CH_3Cl$ was added and the culture was incubated in the shaker for an additional 30 min. Phage lysate was centrifuged at 10,000 x g, for 10 min, at $4^oC$. The phage preparation was titered using strain LE392, at $37^oC$.

Conjugation

The F' complementation analysis and Hfr-mediated interrupted mating experiments were performed as described previously (74) with the E. coli F' kit and Hfr kit obtained from Dr. B. Bachmann, E. coli Genetic Stock Center.

Transformation

Transformation experiments were carried out using the calcium chloride method (70) with some modifications. Overnight LB culture of the appropriate strain was inoculated (1% vol/vol) into 10 ml of fresh LB. The culture was incubated at $30^oC$ with aeration until it reached a density of about 1 x $10^8$ CFU/ml. The culture was shifted to $37^oC$, for 30 min. Cells were harvested by centrifugation at 3,500 x g for 5 min at room temperature. The pellet was washed once with 5 ml of 0.1 M NaCl and resuspended in 5 ml of 0.1 M $CaCl_2$. After 20 min of $CaCl_2$ treatment, the cells were harvested and resuspended in 1 ml of 0.1 M $CaCl_2$. The suspension was kept in ice. Approximately 40-50 ng of plasmid DNA were added to 0.2 ml of the chilled cell suspension which were previously mixed with 10 ul of

dimethyl sulfoxide. The transformation mixture was incubated in ice, for 20 min, followed by heat treatment ($42^{o}C$) for 2 min. The tube contents were incubated in ice for an additional 10 min. One ml of LB was added to the mixture. After 1 to 2 h of incubation at $37^{o}C$, cells were centrifuged at room temperature and resuspended in a small volume of LB. Transformants were selected using appropriate selective media.

Plasmid Preparation

Small scale plasmid isolation was routinely carried out by following the alkaline lysis method described by Maniatis et al. (72). Large scale preparation of plasmids were prepared as described below.

The E. coli strain carrying appropriate plasmid to be isolated was grown aerobically in 1 liter of LB. Cells at the stationary phase of growth were harvested by centrifugation at 5,000 x g, for 10 min, at $4^{o}C$. Cell pellet was resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing glycerol (25% final concentration). Lysozyme was added to a final concentration of 1 mg/ml and the mixture was incubated, at room temperature, for 10 min. Protease was added to a final concentration of 1 mg/ml and followed by the addition of 10 ml of Triton solution, containing 0.1% Triton-X-100, 50 mM Tris-HCl (pH 8.0) and 50 mM ethylenediaminetetracetic acid. Ribonuclease (2 mg/ml, heat treated at $95^{o}C$ for 2 min to inactivate DNase) was

added to the suspension to a final concentration of 100 ug/ml. The lysis mixture was mixed gently and incubated, at room temperature, for an additional 10 minutes. The lysate was centrifuged at 20,000 x g, for 1 h, at 4°C and the supernatant was collected. Cesium chloride was added to the lysate at a final concentration of 0.93 g/ml. After adding ethidium bromide (200 ug/ml final concentration), the solution was centrifuged in a Type-55 rotor, in a Beckman ultracentrifuge, at 50,000 rpm, for 18 h, at 18°C. Plasmid band was collected from the cesium chloride-ethidium bromide gradient using a #21 hypodermic needle inserted into the side of the centrifuge tube. Ethidium bromide was removed from the plasmid-containing DNA solution by extracting with 1-butanol saturated with Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The extraction procedure was repeated twice more after the pink color disappeared from the aqueous solution. After addition of 2 volumes of sterile deionized water, plasmid DNA was precipitated with 2 volumes of 95% ethanol and was kept at -20°C for 18 h. The precipitated plasmid DNA was harvested by centrifugation at 10,000 rpm, for 60 min, at 4°C in the Sorvall SS-34 rotor. The resultant DNA pellet was washed by adding 5 ml of 70% ethanol and then inverting the centrifuge tube several times. The washed DNA pellet was collected by centrifugation for 30 min at 10,000 rpm, at 4°C, in the same centrifuge rotor and dried with gentle air flow. The dried plasmid DNA pellet was resuspended in a small volume of

deionized water. Concentration of DNA in the plasmid preparation was determined either by running an agarose gel with predetermined DNA standards or by measuring the optical density of the solution at 260 nm and 280 nm as described previously (72).

Manipulation of Plasmids

A physical map of recombinant plasmids containing E. coli chromosomal DNA insert, based on restriction endonuclease digestion, was constructed by using previously published procedures (24). Conditions of restriction endonuclease digestion and ligation of DNA by T4 DNA ligase were either as described by Davis et al. (24) or as recommended by the manufacturer of the enzyme.

Agarose Gel Electrophoresis

DNA fragments were separated in 0.7% agarose gels in Tris-borate buffer [89 mM Trizma base, 89 mM boric acid, 2 mM EDTA (pH8.0)] at 9 V/cm for 1-mm analytical vertical gels or at 4 V/cm for preparative horizontal gels. After visualization with ethidium bromide and UV light, DNA-bands were compared with those of lambda DNA fragments obtained after digestion with HindIII. DNA fragments were recovered either from low-melt agarose gel by cutting out the restriction fragments or from preparative gels by electroelution followed by extensive phenol and

phenol/chloroform extraction, as described by Maniatis et al. (72).

Random Mutagenesis of E. coli Chromosome by lac Fusion

E. coli strain BW545 was mutagenized with lambda placMu 53 and lambda pMu 507 (15) to construct random lac operon fusions into the E. coli chromosome as described below. Actively growing E. coli cells, strain BW545, in LB broth containing 0.3% of maltose ($5 \times 10^8$ cfu/ml) were harvested and resuspended in an equal volume of 10 mM $MgSO_4$ solution. To the cell suspension of approximately $10^9$ cells, phage lysates of lambda placMu 53 and lambda pMu 507 were added at a final multiplicities of infection of 0.1 and 1.0, respectively. After 30 min of phage adsorption, at 37°C, 5.0 ml of LB was added to the mixture, to dilute out the uninfected phages. Cells were then sedimented and the resulting cell pellet was resuspended in 1.0 ml of LB. The mutagenized cell suspension was plated on LB plus kanamycin medium and the mutants were selected as $Km^r$ colonies.

Construction of Tn10 Pool

To facilitate strain constructions, random transpositions of Tn10 into the chromosome of E. coli strain MC4100 were obtained using lambda phage NK561 (32), by the method of Foster et al. (32), with some modifications.

Strain MC4100 was grown to mid-log phase ($5 \times 10^8$ cfu/ml) in LB containing 0.4% maltose. Cells were harvested by centrifugation, at room temperature and resuspended in 10 mM $MgSO_4$. Phage lambda NK561 was added to the cell suspension at a multiplicity of 1.0 and allowed to adsorb for 30 min, at room temperature. Cells were sedimented by centrifugation and the resulting cell pellet was resuspended in 1.0 ml of LB. The infected cell suspension was plated on LB plates containing tetracycline and the plates were incubated at 37°C. About 5,000 tetracycline resistant colonies were selected and pooled. Phage P1 grown in the pooled cell population was used for further experiments.

## Construction of Strains Carrying Transposon Tn10 Close to the Various Mutations of Interest in this Study

To facilitate construction of multiple mutant strains carrying different mutations of interest used in this study, transposon Tn10 insertions near the gene of interest were isolated by the following general procedure.

Phage P1 grown in the Tn10 containing pool of cells was transduced into a mutant strain for example, hupA, and tetracycline-resistant transductants were selected and screened for $hupA^+$ genotype. The $hupA^+$ transductant was used as a donor strain for the second transduction of the original mutant strain, selecting for Tn10 and hupA genotypes. The resulting transductants were examined for linkage between hupA and Tn10. A strain which carried Tn10

at reasonably close distance to the hupA gene, measured as high transduction frequency, was used as a donor strain to transduce the hupA mutation into other genetic backgrounds, as needed.

## Mutagenesis of Bacteriophage P1 Transducing Particles

In order to isolate an auxotrophic mutation which may reside within a co-transducible distance to the gene of interest, a localized mutagenesis procedure was effectively used with the aid of phage P1 mutagenized appropriately with hydroxylamine, as described below.

Phage P1 grown in strain SE1319 (Fh1C$^+$, Tn10) was extracted with chloroform 3-times to remove excess cell protein which may harden the phage pellet during purification. Phage from an 110 ml lysate ($6.6 \times 10^9$ pfu/ml) was concentrated by centrifuging at 48,000 x g, for 1 h, at 4$^o$C. The phage pellet was resuspended very gently in 2.5 ml of LB containing EDTA (2 mM) and $CaCl_2$ (10 mM). The concentrated P1 phage preparation ($2.8 \times 10^{11}$ pfu/ml) was mutagenized with hydroxylamine by following the procedure described by Murgola and Yanofsky (75) with some modifications. Two ml of the resuspended phage were added to 18 ml of 0.45 M hydroxylamine-hydrochloride (adjusted to pH 6.0 with NaOH) containing 2 mM EDTA and 10 mM $CaCl_2$, and the mixture was incubated at 37$^o$C for 18 h. The phage was sedimented and the pellet was resuspended in 1 ml of P1 diluent. During the 18 h mutagenesis period, the viable

phage population decreased by $1.8 \times 10^{4}$. These conditions gave results similar to Hong and Ames (46), particularly with regard to the rate of inactivation of phage and the increase in co-transducible mutations. The mutagenized phage was stored at $4^{o}C$ with few drops of chloroform.

## Transposon Tn5 Mutagenesis of fhlC Gene in Recombinant Plasmid

For the purpose of mapping fhlC gene in the chromosomal DNA insert of fhlC recombinant plasmid and for isolating a mutant strain carrying Tn5-induced fhlC mutation in its chromosome, fhlC gene cloned in recombinant plasmid was mutagenized with transposon Tn5, as described below.

A difficulty encountered in transposon Tn5 mutagenesis of cloned gene in multicopy plasmids was the low frequency with which Tn5 transposes itself into the plasmid DNA. This problem was overcome by using a supF mutant host strain. Lambda NK421 requires both supE and supF mutations in its host strain for multiplication. Thus, we reasoned that supF host strain would increase the copy number of the lambda DNA in its cytoplasm, without killing the host cell, which would increase the frequency of Tn5 transposition. Indeed the frequency of Tn5 transposition to the plasmid in MBM7014 (supF) was about 100-fold higher than in $supF^{+}$ strain (BW545). For this reason, strain MBM7014 (supF) was used as a host strain for transposon Tn5 mutagenesis of plasmid-born fhlC gene in this study.

An fhlC gene containing plasmid (pSE1004) was transformed into strain MBM7014 (supF), and the resulting transformants were mutagenized with transposon Tn5 using lambda NK421 as described below. A transformant containing plasmid pSE1004 was grown in 20 ml of LB plus 0.4% maltose medium to mid-log phase (3 x $10^8$ cfu/ml). Cells were harvested at room temperature and resuspended in 1 ml of 10 mM $MgSO_4$ solution. The cells were infected with lambda NK421 (Tn5) at an M.O.I. of 10. After 30 min of incubation, at room temperature, the infected cells were sedimented by centrifugation and washed with 5 ml of LB in order to remove uninfected phage particles and resuspended in 10 ml of LB medium. Two ml of this cell suspension were inoculated into 10 ml of fresh LB medium containing 0.4% glucose and 10 mM Na-citrate in 125 ml Erlenmeyer flask and incubated at 37°C with shaking for 30 min. After 30 min at 37°C, the incubation temperature was shifted to 30°C. At 30°C, the lambda repressor becomes active and prevents phage growth. After 1 h incubation at 30°C, kanamycin and ampicillin were added at a concentration of 50 ug/ml and 100 ug/ml, respectively. When the culture reached the maximum cell density, usually after 18 h of incubation, 1.5 ml of the culture from the flask were removed and the plasmid DNA was isolated using the mini-plasmid preparation method. The plasmid DNA was used to transform strain SE1187 (fhlC, $Km^s$), and transformants containing the plasmid with transposon Tn5 were selected on LB agar containing kanamycin and

ampicillin. Plasmids carrying Tn5 in the *fhlC* gene were identified as described in the "Results" section.

## RESULTS

### Isolation and Chracterization of Formate Hydrogenlyase Defective Mutants

Formate hydrogenlyase, the enzyme complex involved in the fermentative $H_2$ production, is regulated by $O_2$ and $NO_3^-$ (28, 106). Recent studies of three different lac fusion strains, M9s (fdhF::lac) (84), M3s (hyd::lac) (111) and GW5305 (ant::lac) (110), revealed that the regulation of three independent genes, producing components of the FHL complex, is similar to FHL. These similarities in the regulation of the activity of the components of formate hydrogenlyase raised the possibility that E. coli may regulate this pathway, from formate to hydrogen, as one metabolic unit (regulon). Since FDH2 and FHL-hydrogenase are components of the formate hydrogenlyase system, a single gene product which is unique for this pathway could conceivably control the synthesis of these two enzymes as well as the intermediate electron transport components. If such a regulatory mechanism exists in the cell, it should be possible to isolate a mutant strain defective in the regulatory gene. Isolation and characterization of a mutant strain defective in such a gene would be of great importance

for understanding the regulation of $H_2$ evolution by an anaerobic cell.

Direct selection of mutant strains defective in FHL-hydrogenase activity would be difficult, because, although the FHL system is involved in degrading formate, mutant strains defective in FHL activity do not provide a selectable phonotype. If mutant strains defective in $H_2$-dependent BV reduction were isolated (62), these mutant strains would be defective in HUP-hydrogenase activity or lack both hydrogenase activities and would not necessarily be limited to the FHL-hydrogenase activity alone (62).

For these reasons, the capacity of formate dehydrogenase 2 to reduce BV, using formate as electron donor, can be used for initial screening of a large number of mutagenized cells for identification of mutants defective in FDH2. These $Fdh^-$ mutants can be further screened for the presence of hydrogenase and $H_2$-uptake activites. The mutant strains defective in FDH2 activity and normal for HUP activity can be tested for the presence of FHL-hydrogenase activity. A mutant strain lacking both FDH2 and FHL-hydrogenase activities would be considered as a regulatory mutant since the mutation abolished two separate enzyme activities, the genes for which are not known to be linked.

With this rationale for the mutant isolation, strain BW545 was mutagenized with an operon fusion, generating lambda phage, lambda plac Mu 53, as described in the

"Materials and Methods" section. The mutagenized cell population was plated on LB agar containing kanamycin and mutant strains were selected as kanamycin-resistant colonies under aerobic conditions. These $Km^r$ colonies were transferred to LB agar by replica plating method and incubated at room temperature, under anaerobic conditions, in order to induce formate dehydrogenase 2 activity. After colonies developed, these were overlayed with soft agar (3.Ø ml/plate) containing formate dehydrogenase 2 assay mixture. Mutant strains defective in FDH2 activity were selected as colonies which reduced BV at a very low rate. Four mutant strains lacking FDH2 activity were isolated from several hundred mutants. These four $Km^r$ and $FDH2^-$ mutant strains were named SE1lØØ, SE1lØ1, SE1lØ2 and SE1lØ3.

## Biochemical Characterization of the Formate Hydrogenlyase Defective Mutants

Biochemical characteristics of the four $FDH2^-$ mutant strains, grown in LB, anaerobicaly, were determined by measuring formate dehydrogenase activities (both FDH1 and FDH2), using crude extracts and hydrogenase and hydrogen uptake (HUP) activities, using whole cells. The results of these experiments are presented in Table 2. All four mutant strains produced very low or undetectable levels of both formate dehydrogenase activities. Formate hydrogenlyase activity was not detected in any of the four mutant strains.

Table 2. Biochemical characteristics of E. coli mutant strains grown in LB medium

| Strain | Enzyme Activity* | | | | | |
|---|---|---|---|---|---|---|
| | FDH2 | FDH1 | HYD | FHL | HUP +BV | HUP +Fumarate |
| BW 545 | 29.0 | 22.0 | 725 | 14 | 816 | 250 |
| SE-1100 | 0.4 | 1.3 | 746 | UD | 962 | 265 |
| SE-1101 | 1.2 | 0.3 | 609 | UD | 889 | 277 |
| SE-1102 | UD | 0.9 | 525 | UD | 669 | 157 |
| SE-1103 | UD | 1.0 | 769 | UD | 828 | 291 |

* nmol / h x (mg cell protein)$^{-1}$ ; UD - Undetectable

However, these strains produced normal levels of hydrogenase activity. Hydrogen uptake (Hup) activities of these mutant strains, both BV-dependent and fumarate-dependent, were comparable to wild type levels. These results show that the four FDH2 defective mutant strains, grown in LB medium, are similar.

It is possible that the lack of FDH2 and FHL activities in these mutant strains may be a consequence of the absence of formate in the LB medium, a substrate known to have a positive effect on FHL production (84). In order to evaluate this possibility, these four mutant strains were further analyzed, using the cultures grown in three different growth media which are known to supply formate to the cells, either produced internally from glucose or provided externally: glucose minimal medium, LB + glucose (Ø.3%) and LB + formate (Ø.1%). Changing the growth medium did not alter the phenotype of three of the four mutant strains (SE11Ø1, SE11Ø2, and SE11Ø3; Table 3). These results raised two possibilities about the nature of the defect(s) in these mutant strains. One possibility is that the defect is probably in the structural component of FDH2. A second possibility is that the defect is in a regulatory gene which is essential for the production of FDH2 and FHL acitivities in all these growth conditions. On the other hand, strain SE11ØØ behaved differently from the other three mutant strains and produced normal levels of FHL activity in glucose minimal medium. Formate dehydrognease 2 activity

Table 3. Biochemical characteristics of $Fdh^-$ strains grown in different media

| Enzyme activity* | Strain | | | | |
|---|---|---|---|---|---|
| | BW 545 | SE-1100 | SE-1101 | SE-1102 | SE-1103 |
| **Glucose minimal medium** | | | | | |
| Formate hydrogenlyase | + | + | - | - | - |
| Formate dehydrogenase 2 | + | + | - | - | - |
| Hydrogenase | + | + | + | + | + |
| **LB+glucose (0.3%) medium** | | | | | |
| Formate hydrogenlyase | + | - | - | - | - |
| Formate dehydrogenase 2 | + | - | - | - | - |
| Hydrogenase | + | + | + | + | + |
| **LB+formate (0.1%) medium** | | | | | |
| Formate hydrogenlyase | + | - | - | - | - |
| Formate dehydrogenase 2 | + | - | - | - | - |
| Hydrogenase | + | + | + | + | + |

* nmole / h x (mg cell protein)$^{-1}$ ; + denotes normal parent levels; - denotes that the activity is below detectable levels.

was detectable in strain SE1100 when grown in glucose minimal medium. Since this mutant strain did not produce formate dehydrogenase 2 and FHL activities when grown in LB + formate medium, the production of FDH2 by glucose minimal medium-grown strain SE1100 is not due to the presence of formate in the growth medium. This variability in induction of FHL and FDH2 activities of the mutant strain, SE1100, in different culture media, demonstrates that the FHL defect observed in this mutant is not in a structural component of the FHL complex but rather in a gene controlling the production of FHL. Based on this reasoning, strain SE1100 was chosen for further studies.

## Strain SE1100 is also Defective in FHL-hydrogenase Activity

Since the strain SE1100 did not produce formate dehydrogenase activity in LB medium, the lack of FHL activity may be a consequence of this defect. On the other hand, this mutant may affect a requlatory gene abolishing the production of FDH, FHL-hydrogenase and electron transport proteins involved in FHL. If the second possibility is correct, the hydrogenase, detected in strain SE1100, grown in LB medium, should be involved only in the HUP pathway and not in FHL. A genetic approach was used to distinguish between the two hydrogenase activities.

It has been established previously, that a gene responsible for production of HUP-hydrogenase activity

(hupA) mapped at 65 min in E. coli chromosome (8, 62). Biochemical characterization of these hupA mutants showed that these strains failed to produce HUP-hydrogenase isoenzyme, but produced FHL-hydrogenase isoenzyme (unpublished data). If the hydrogenase activity present in the LB culture of strain SE1100 represents only the HUP-hydrogenase activity, then, a hupA derivative of strain SE1100 would not produce any detectable hydrogenase activity ($^3H_2$ exchange) in LB medium.

In order to test this possibility, double mutant strains carrying the defect in strain SE1100 and hupA mutation were constructed by transducing the hupA mutation from strain SE1261 into strain SE1100. One of the resulting double mutant strains, SE1455, was grown in LB medium and tested for the presence of hydrogenase activity. As shown in Table 4, introduction of hupA mutation into strain SE1100 reduced the levels of hydrogenase activity from 746 units to 56 units and HUP activity to undetectable levels. Most of the hydrogenase activity (92%) and all the HUP activity were eliminated by transferring the hupA mutation into strain SE1100. These results clearly show that the gene altered in strain SE1100 is required for production of FHL-hydrogenase activity as well as FDH2 activity.

Because of the unique pleiotropic characteristics of this mutant strain and a new hitherto unidentified map location in E. coli chromosome (based upon genetic data presented in a different section), the gene altered in

Table 4. Biochemical activities of strain SE1100 and its hupA derivative (SE1455) grown in LB medium.

| Strain | Relevant genotype | Enzyme activity* | | |
|---|---|---|---|---|
| | | FHL | HYD ($^3H_2$ ex.) | HUP ($H_2$/BV) |
| BW545 | WT | 14 | 725 | 816 |
| SE1100 | fhlC | UD | 746 | 962 |
| SE1455 | fhlC, hupA | UD | 56 | UD |
| SE29 | hupA | 36 | 661 | UD |

* nmol / h x (mg cell protein)$^{-1}$; UD - undetectable

strain SE1100 was named fhlC, meaning defective in formate hydrogenlyase, following after fhlA (90) and fhlB (unpublished data)

Molybdenum Suppresses the fhlC Mutation

In order to identify the component(s) present in glucose minimal medium which facilitated the induction of FHL activity by SE1100, LB/glucose (0.3%) medium was supplemented with various medium components normally present in glucose minimal medium. Glucose minimal medium contains $PO_4^{-3}$, NaCl, $(NH_4)_2SO_4$, $MgSO_4$, and trace metals (Mo, Se and Fe). The mutant strain was grown in LB/glucose medium containing these components and FHL activity of these cells was measured. Strain SE1100 produced FHL activity in LB/glucose medium only when supplemented with trace minerals. The effect of these minerals on the induction of FHL activity was further analyzed by measuring the FHL activity in the mutant strain grown in LB/glucose medium supplemented with these trace minerals in a final concentration of 1 mM (Table 5). Addition of sodium molybdate to the growth medium led to production of FHL activity by the mutant strain. Sodium selenite and ferrous sulfate addition had no effect. These data show that molybdate is a specific requirement for FHL production by strain SE1100.

In order to determine the molybdate concentration required to overcome the fhlC effect on production of

Table 5. FHL activities of strains BW545 and SE1100 grown in LB/glucose medium with different trace mineral supplements

| Supplement* | FHL activity | |
|---|---|---|
| | BW545 (WT) | SE1100 (*fhlC*) |
| None | 600 | UD |
| Mo | 580 | 381 |
| Se | 429 | UD |
| Fe | 415 | UD |

* Supplements were added to LB/Glucose (0.3%) medium at an 1 mM concentration.

formate hydrogenlyase, the FHL activity profile of both wild type and strain SE1100, grown in LB/glucose with increasing levels of molybdate, was determined and presented in Fig. 1. Wild type strain, BW545, produced 183 units of FHL activity even in a medium without molybdate addition. This activity slightly increased up to 25 uM of molybdate from 183 units to 203 units. Addition of molybdate at concentrations higher than 25 uM had no effect. The mutant, strain SE1100, did not produce any detectable FHL activity up to 10 uM molybdate in the medium. A sharp increase in FHL activity can be observed between 10 uM and 25 uM molybdate supplement. Beyond 25 uM molybdate, the FHL activity of the cells increased at a lower rate as the molybdate concentration in the medium increased. At 100 uM molybdate, FHL activity produced by the mutant was about 90% of the maximum wild type levels. About 25 uM molybdate was required by the mutant strain to produce 50% of the wild type level of FHL activity in LB/glucose medium (Fig. 1).

In glucose minimal medium, which contains 46 uM molybdate, the mutant strain produced FHL activity which are comparable to the wild type values (Table 3). However, at the same concentration of molybdate, in LB/glucose medium, the mutant strain produced only 60% of the wild type level. These results show that the optimum concentration of molybdate needed for production of FHL activity is dependent on the medium composition. In order to test this possibility, an FHL activity profile of cultures grown in

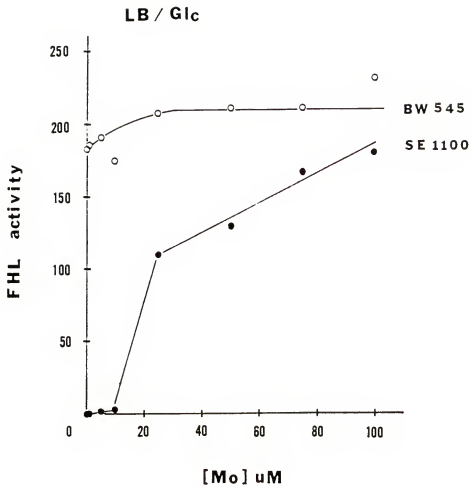



Fig. 1. Effect of addition of molybdate to the growth medium on production of formate hydrogenlyase (FHL) activity by E. coli mutant strain SE1100 and its parent (BW545) grown in LB/glucose (0.3%) medium. FHL activity is expressed as nmol $H_2$ produced / min x (mg cell protein)$^{-1}$.

molybdate-deficient glucose minimal medium supplemented with various concentrations of molybdate was determined (Fig. 2). In wild type, without molybdate addition to the growth medium, there was maximum FHL activity and this activity remained at the same level as molybdate concentration increased, up to 100 uM, in the growth medium. The mutant strain did not produce FHL activity if molybdate was not added to the medium. With 1 uM molybdate supplement, the mutant produced about 80% of wild type level of FHL activity. At 5 uM molybdate concentration, strain SE1100 produced about 90% of the wild type level and beyond that concentration, the activity remained unchanged, up to 100 uM. The molybdenum concentration, required for the fhlC mutant strain to produce 50% of wild type level of FHL activity, was determined using very low concentrations of molybdenum added to the growth medium (Fig. 3), and it was found to be about 0.5 uM. Therefore, in order to produce 50% of the wild type level of FHL activity, the mutant (fhlC) strain required 50-fold less molybdate in the glucose minimal medium than in LB/glucose medium.

## Selenium Suppresses Mo-dependent FHL Activity in fhlC Mutant Strain

It has been reported, using highly purified growth medium, that as little as 10 nM molybdate in growth medium is sufficient for production of maximum formate dehydrogenase activity in E. coli (86). This observation

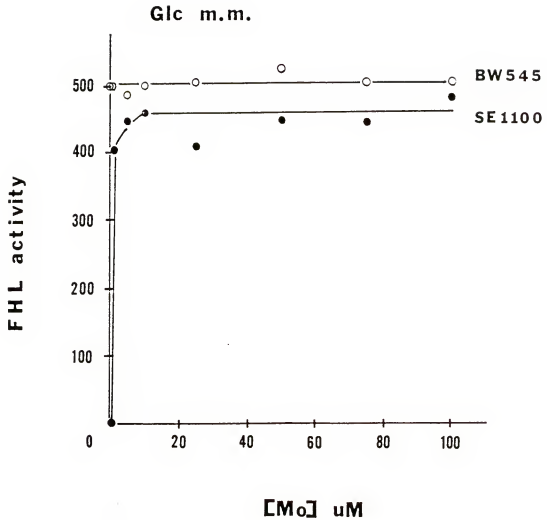



Fig. 2. Effect of addition of molybdate to the growth medium on production of FHL activity by E. coli fhlC mutant strain SE1100 and its parent (BW545) grown in molybdate-deficient glucose minimal medium. FHL activity is expressed as nmol $H_2$ produced / min x (mg cell protein)$^{-1}$.

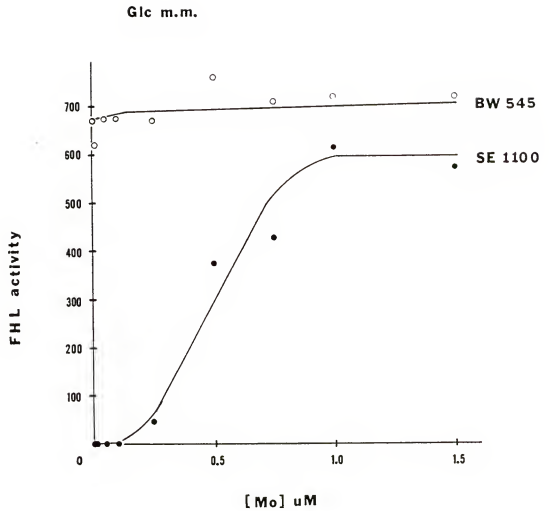



Fig. 3. Effect of addition of molybdate to the growth medium on production of FHL activity by E. coli fhlC mutant strain SE1100 and its parent (BW545) grown in molybdate-deficient glucose minimal medium. FHL activity is expressed as nmol $H_2$ produced / min x (mg cell protein)$^{-1}$.

suggests that wild type E. coli cell has a very efficient molybdenum utilization system. The results presented in Fig. 1 and Fig. 3 suggest that the fhlC mutant, strain SE1100, may lack this highly efficient molybdenum utilization system. In the fhlC mutant, the added molybdate may be transported through a second, low affinity transport system or could be transported through some other ion transport system.

In order to distinguish between these alternatives, FHL activity was measured in the fhlC mutant strain grown in molybdenum (25 uM)-fortified LB/glucose medium which was also supplemented with other minerals at a final concentration of 1 mM. Addition of nickel, magnesium, iron, manganese, cobalt, copper or zinc to the growth medium did not affect the molybdate-dependent FHL activity. However, presence of 1 mM selenite in the growth medium completely abolished the molybdenum-dependent FHL activity of the mutant strain, although the wild type strain, grown in the same medium, produced more than twice the FHL activity (Table 6) in the presence of selenite and molybdate.

This inhibitory effect of selenite on the production of molybdenum-dependent FHL activity by strain SE1100 was further examined, by measuring the FHL activity in both wild type and mutant strains grown in molybdenum-containing (25 uM) LB/glucose medium with various [Se]/[Mo] ratios (Fig. 4). Since high concentrations (>1 mM) of selenite inhibited growth, the LB/glucose medium was supplemented with only 25

Table 6. Effect of selenite on molybdate-dependent FHL activity in *E. coli fhlC* mutant strain

| Strain (Genotype) | Mo | Se | FHL activity* |
|---|---|---|---|
| BW545 (WT) | - | - | 166 |
| | + | - | 149 |
| | + | + | 334 |
| SE1100 (*fhlC*) | - | - | UD |
| | + | - | 219 |
| | + | + | UD |

LB/Glucose (0.3%) medium was supplemented with 25 uM molybdate (Mo) and 1 mM selenite (Se) as indicated, and used to culture the organism.

* nmol $H_2$ produced / min x (mg cell protein)$^{-1}$;
UD - undetectable

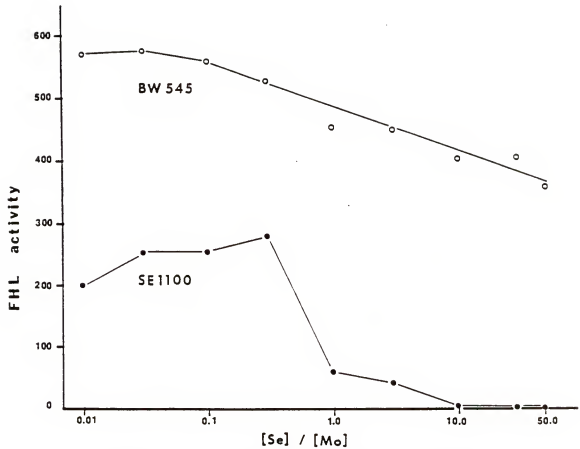



Fig. 4. Effect of increasing selenite concentration on FHL activities of E. coli strain BW545 (WT) and SE1100 (fhlC). Cells grown in LB medium containing glucose (0.3%) and molybdate (25 uM) supplemented with different concentrations of sodium selenite were used in the assay. FHL activity is expressed as nmol $H_2$ produced / min x (mg cell protein)$^{-1}$.

uM molybdate, although this concentration yielded only about 50% of the wild type level of FHL activity (Fig. 1). Wild type strain (BW545) produced maximum FHL activity when the medium contained a [Se]/[Mo] of Ø.Ø1 and Ø.Ø3. The activity declined very slowly as selenium concentration increased. Even at a ratio of 5Ø, this strain produced 65% of the maximum activity. However, the mutant strain showed a gradual increase in FHL activity between the ratios of Ø.Ø1 and Ø.3. Maximum FHL activity was produced by the mutant strain when grown in a medium containing about 7.5 uM selenite, at this fixed molybdate concentration. At higher selenite concentrations, the FHL activity in the mutant strain rapidly declined. The FHL activity was not detectable when selenite was present at 1Ø- fold higher concentration than molybdate. This inhibitory effect of selenite on production of FHL activity by the mutant strain suggests that molybdate is probably transported by the cell through a selenite transport system. At high selenite concentrations, selenium probably blocks molybdenum transport/utilization. Because of the lack of molybdate, the mutant cell probably can not produce FHL activity.

## Genetic Mapping of fhlC Gene

A chlorate-resistant mutant strain carrying a mutation (chlD) at 17 min in E. coli chromosome has been reported to be deficient in formate dehydrogenase activity as well as all other molybdoenzyme activities (35). The effect of the

chlD mutation can also be suppressed by addition of high concentrations (1 mM) of molybdenum to the growth medium. These phenotypic characteristics of the chlD mutants are similar to those of the fhlC mutant described in the previous section. To determine whether these two mutations reside in the same gene, the fhlC gene was mapped.

As a first step in mapping the gene, the location of the lambda placMu in the fhlC gene was confirmed. This was demonstrated by transducing kanamycin resistance from the strain (SE1100), via phage P1, into a recipient strain MC4100. When grown in LB/glucose medium, all $Km^r$ transductants lacked FHL activity. These results show that the mutation in strain SE1100 is indeed produced by a gene fusion (lacMu).

## Hfr-mediated Conjugation

The approximate map location of the fhlC mutation was determined by Hfr-mediated conjugation. For these experiments, the fhlC mutation was transduced from strain SE1100 to strain Hfr3000 and $Km^r$ transductants were selected. One of the transductants (SE1119) was used as a donor strain for an Hfr-mediated interrupted mating experiment using MC4100 as a recipient strain. Time of entry of the fhlC::lac ($Km^r$) was determined by scoring the time of entry of the $Km^r$ gene. Based upon this interrupted mating experiment, the fhlC mutation was located approximately at 68 min in E. coli chromosome. This map

location of the fhlC gene distinguished this gene from the chlD gene which mapped at 17 min.

In further experiments designed to determine the location of fhlC gene more accurately, the fhlC::lambda placMu fusion strain (SE1100) conferred two difficulties. The major difficulty is the presence of a large piece of lambda placMu DNA in the fhlC gene, which by decreasing the recombination efficiency, lowered the transduction frequency. Since a large number of transductants are required to determine co-transduction frequencies with nearby genetic markers accurately, this insert DNA needs to be removed. The second problem encountered was related to the availability of only a few genes which are known to map in the region of E. coli chromosome between 65 and 69 min and thus be useful for mapping purposes.

## Transductional Analysis

### Construction of a Strain carrying Transposon Tn10 close to fhlC Gene

To facilitate the identification of fhlC map location, an attempt was made to isolate auxotrophic mutant strains which carry mutations within a co-tranducible distance of the fhlC gene. In order to aid the isolation of such auxotrophic mutant strains, transposon Tn10 was inserted near the fhlC gene and the tetracycline resistance, conferred by the Tn10, was used as a co-transducible marker

for strain construction by localized mutagenesis method as decribed in the "Materials and Methods" section.

The first step in this strain construction required that the transposon Tn10 be inserted near the fhlC gene. For this purpose, phage P1 lysate propagated in the "MC4100-Tn10 pool" (see "Materials and Methods" section for details) was used to transduce strain SE1100 (fhlC). Tetracycline-resistant transductants were selected and tested for kanamycin resistance. Among a total of 233 $Tc^r$ transductants, three independent $Km^s$ colonies (potential $fhlC^+$ transductants) were isolated. These 3 $Tc^r$ and $Km^s$ tranductants were tested for FHL activity after anaerobic growth in LB/glucose medium. All three transductants produced FHL activity. Since the frequencies for random Tn10 transposition and an independent transduction for $fhlC^+$ were expected to be low, the transductants which acquired both tetracycline resistance and kanamycin sensitivity simultaneously were assumed to have Tn10 in a gene near the wild type fhlC gene in the donor chromosome. None of the transductants were found to be auxotropic mutants. One of the three Tn10-carrying strain was designated as SE1130 ($Fhl^+$, Tn10) and used for further strain construction.

## Isolation and Characterization of auxotrophic Mutations which are near the fhlC Gene

In order to isolate mutant strains carrying auxotrophic mutations near the fhlC gene, a different

method, involving localized mutagenesis, was used. For this experiment, phage P1 was grown in strain SE1130. The resulting P1 lysate was mutagenized with hydroxylamine. The mutagenized phage population was used to transduce tetracycline resistance into strain MC4100 at a multiplicity of infection of 0.01 (based upon the phage titer after mutagenesis). Tetracycline resistant transductants were selected in LB medium and auxotrophs present in this population were isolated. Since the genetic material received by these transductants was previously mutagenized, there will be a high probability that the altered gene in these mutant strains is within co-transducible distance with the fhlC gene.

Among a total of 845 independent $Tc^r$ transductants screened, 4 strains failed to grow in glucose minimal medium. In order to identify the auxotrophic requirements of these four mutant strains (SE1156, SE1157, SE1158 and SE1159), they were grown in glucose minimal medium with various nutritional supplements. Strains SE1156 and SE1159 did not grow with any of the nutrient supplements added to the minimal medium either singly or in combination, although these strains grew well in LB. The auxotrophic requirements of these strains were not investigated further. The other two strains, SE1157 and SE1158, grew well in glucose minimal medium with a supplement of either glutamine, glutamate or proline. Further analysis of the auxotrophic requirement of these mutants showed that they require alpha-ketoglutarate

(alpha-KG) for growth (Table 7). Since citrate or isocitrate failed to support growth in minimal medium, the defect in these mutant strains is probably in isocitrate dehydrogenase activity. For this reason, the genotype of these mutants was designated as icd. These icd mutants were used for fhlC mapping experiments.

F' Complementation Analysis

In order to determine approximate map location of the fhlC mutation, the icd mutations were mapped by F' complementation. Among all the F' elements tested, extending over most of the E coli genome, only F'122 which covers between 65 and 69 minutes of the E. coli chromosome complemented both of these mutant strains for $Icd^+$ character (Table 8). Two other F' elements which carry chromosomal DNA which overlap with F'122, F'141 (69 min - 76 min) and F'116 (60 min - 65 min), did not complement the mutations in either mutant. Based on F' analysis, icd gene was mapped between 65 and 69 min in E. coli chromosome.

The F' complementation analysis of fhlC mutation was performed by constructing a double mutant strain carrying both icd and fhlC mutations. The icd mutation was transduced from strain SE1157 (icd, zgg-3::Tn10) to strain SE1100 and $Tc^r$ transductants were selected. These $Tc^r$ transductants were tested for Icd phenotype. Several mutant strains carrying both icd and fhlC mutations were selected for further analysis.

Table 7. Identification of auxotrophic requirement of strains which carry mutation near fhlC gene

| Medium | Supplement | Growth | |
|---|---|---|---|
| | | SE1157 | SE1158 |
| LB | None | + | + |
| Glc-mm* | None | - | - |
| | + alpha KG | + | + |
| | + isocitrate | - | - |
| | + citrate | - | - |

* Glc-mm: glucose minimal medium

Table 8. F' complementation analysis of the auxotrophic mutation (*icd*) near *fhlC* gene

| Strain | Icd phenotype | | |
|---|---|---|---|
| | F'141 | F'122 | F'116 |
| SE1157 | - | + | - |
| SE1158 | - | + | - |

One of the double mutant strains, SE1433 (icd, zgg-3::Tn10, fhlC::lambda placMu), was used for F' complementation analysis of fhlC mutation. The F'122 was introduced by conjugation into the strain SE1433. Exconjugants were selected as $Icd^+$ colonies. Several of these exconjugants were grown in LB/glucose medium anaerobically and tested for FHL activity. All the exconjugants produced FHL activity. The F' complementation data using one of the exconjugants, SE1448, are presented in Table 9. These F' complementation data, together with Hfr-interrupted mating data, clearly show that the fhlC mutation maps between 65 and 69 min in E. coli chromosome and the icd gene is near the fhlC gene.

## Isolation of a Transposon Tn5 induced fhlC Mutation (fhlC::Tn5)

Since the fhl:lac fusion strain, SE1100, carries a large size of lambda placMu DNA insertion in the fhlC gene, transduction frequency of this fhlC gene with the large insert was not high enough ($10^{-8}$) to produce a sufficiently large number of transductants required to accurately determine co-transduction frequencies with nearby genetic markers. Construction of an fhlC deletion by removing the lambda phage will also abolish the selectable $Km^r$ phenotype. In order to overcome these problems, a mutant strain carrying transposon Tn5 in the fhlC gene in E. coli chromosome was constructed. Transposon Tn5 is a

Table 9. F' complementation analysis of fhlC mutation

| Strain | Relevant genotype | F' element | FHL* |
|---|---|---|---|
| BW545 | WT | None | 132 |
| SE1100 | fhlC | None | UD |
| SE1433 | fhlC, icd | None | UD |
| SE1448 | fhlC, Icd$^+$ | F'122 | 140 |

* nmol $H_2$ produced / min x (mg cell protein)$^{-1}$

comparatively small DNA element (5.5 kb) and provides $Km^r$ phenotype also.

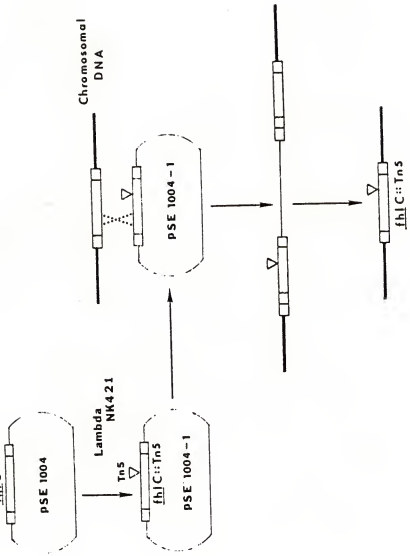
Isolation of fhlC::Tn5 mutant strain utilized the following steps as illustrated in Fig. 5; i) Transposon Tn5 mutagenesis of wild type fhlC gene cloned in a plasmid ii) Transferring the fhlC::Tn5 mutation from the plasmid to the chromosome in a polA mutant strain and iii) transducing fhlC::Tn5 from polA to $polA^+$ strain.

Construction of a kanamycin-sensitive derivative of strain SE1100.

The fhlC::lac fusion strain, SE1100, carries neomycin phosphotransferase gene (from lambda placMu) conferring kanamycin resistance to the cell. In order to replace the lambda-based $Km^r$ gene with transposon Tn5, a kanamycin-sensitive fhlC mutant strain needs to be constructed for selection of plasmids containing Tn5 ($Km^r$) in the fhlC gene in the plasmid. This goal was accomplished by replacing lambda-$Km^r$ phage with another lambda phage P1(209). This phage deleted for lambda att gene can only integrate into host chromosome of a lambda lysogen by recA-dependent homologous recombination (57). Infecting strain SE1100, a lambda placMu ($Km^r$) lysogen, with lambda p1(209) would give rise to lambda exchange and thus $Km^s$ phenotype.

Towards this objective, strain SE1100 was spread on LB agar, and a lambda p1(209) lysate was spotted on the lawn of the bacterial cells and incubated at $37^oC$, overnight.

Fig. 5. Diagrammatic representation of the construction of an *fhl*::Tn5 mutation in the chromosome. A recombinant plasmid (pSE1004) carrying wild type *fhlC* gene (*fhlC*$^+$) was introduced into a *supF* host (MBM7014) and mutagenized with transposone Tn5 using lambda phage NK421. The recombinant plasmid carrying transposone Tn5 induced *fhlC* mutation (*fhlC*::Tn5) was identified in strain SE1187 (*fhlC*, Km$^s$) by selecting Ap$^r$ and Km$^r$ transformants which could not complement for the *fhlC* mutation. The resulting recombinant plasmid carrying the *fhl*::Tn5 mutation was introduced into a *polA* strain (P3478). Recombination can occur between the chromosomal DNA present in the plasmid and the corresponding homologous region in the chromosome. The plasmid integrates were obtained by selecting Ap$^r$ and Km$^r$ colonies. After removal of the drug challenge, Ap$^s$ segregates were obtained when plasmid DNA resolves from the chromosome via homologous recombination. The segregates retain either the original sequence (*fhlC*$^+$) or the altered sequence (*fhlC*::Tn5) in the chromosome. The segregates containing the *fhlC*::Tn5 mutation in the chromosome were selected as Ap$^s$ and Km$^r$ colonies.


fhlC'
pSE 1004
Lambda
NK421
Tn5
fhlC::Tn5
pSE 1004-1
Chromosomal
DNA
pSE 1004-1
fhlC::Tn5

Lysogens were collected from the resulting lysis zone and were tested for $Km^S$ phenotype. One out of a total of 400 independent lysogens tested was found to be sensitive to kanamycin. It was determined that this $Km^S$ strain, SE1187, carryed the fhlC mutantion.

Transposon Tn5 mutagenesis of fhlC gene cloned in a plasmid.

A recombinant plasmid carrying cloned $fhlC^+$ gene, plasmid pSE1004 (described in a later section on "Cloning"), was transformed into strain MBM7014 (supF), and a resulting transformant was mutagenized with transposon Tn5, using lambda NK421, as described in the "Materials and Methods" section. Plasmids were isolated from the mutagenized cells, using small-scale plasmid preparation method. The plasmids were transformed into a kanamycin-sensitive, fhlC mutant, strain SE1187, and $Km^r$ transformants containing Tn5 in the plasmid were selected on LB-agar containing kanamycin and ampicillin. The $Km^r$ and $Ap^r$ transformants were tested for FHL activity after anaerobic growth in LB/glucose medium. The plasmids carrying Tn5 in the fhlC gene will not complement the fhlC mutation in the chromosome and the merodiploids will be $Fhl^-$. Three transformants carrying independent fhlC::Tn5 were isolated as $Fhl^-$ and the plasmids were isolated individually from these transformants. The plasmids were transformed again into strain SE1187 to confirm that the plasmids confer both $Km^r$ and $Ap^r$ characters

and lack intact fhlC gene. One of these plasmids was found to carry fhlC::Tn5 mutation and was used for construction of a mutant strain carrying fhlC::Tn5 mutation in the chromosome.

Transferring fhlC::Tn5 mutation from plasmid to chromosome.

In order to isolate a mutant strain carrying transposon Tn5-induced fhlC mutation (fhlC::Tn5) in the chromosome, the fhlC::Tn5 mutation in plasmid pSE1004-1 was tranferred to the chromosome by following the gene replacement technique described by Gutterson and Koshland (39). Plasmid pSE1004-1 consists of the chromosomal DNA insert in the vector pBR322 which has an Col El replicon. The latter will not replicate in a polA strain, one deficient in DNA polymerase 1 (49). Upon transformation, if the transformants were selected for plasmid-borne characters, only those cells in which plasmid genes integrate into the host chromosome (by homologous recombination) will replicate and produce a colony. The plasmid integrates contain direct nontandem duplication of bacterial sequences separated by vector sequences (39). Therefore, this system would provide a positive selection for incorporation of plasmid genes into the host chromosome.

For these experiments, a polA strain P3478 was transformed with plasmid pSE1004-1. Transformants were selected as $Km^r$ and $Ap^r$ colonies on LB-agar containing

kanamycin and ampicillin. Therefore, the $Km^r$ and $Ap^r$ transformants will have plasmid integrated in the chromosome (plasmid integrate) via homologous recombination between the chromosomal DNA present in the plasmid and the corresponding homologous region in the chromosome (39). In order to obtain segregates which contain altered chromosomal segments (fhlC::Tn5) without the vector sequences, the $Km^r$ and $Ap^r$ transformants were subcultured in LB medium without any antibiotic challenge, for several generations. The cell population grown in LB medium was screened for colonies resistant to kanamycin but sensitive to ampicillin. One such $Km^r$ and $Ap^s$ strain was isolated. This strain was also found to be fhlC, as determined by the absence of FHL activity when grown in LB/glucose medium. The presence of fhlC mutation in the mutant strain was further confirmed by complementing the fhlC mutation to wild type phenotype with plasmid pSE1Ø04 (see "Cloning" section). This strain was named SE1319 and used as fhlC::Tn5 mutant strain for the following mapping experiments.

## Bacteriophage P1-mediated Transduction Analysis of fhlC Mutation with respect to nearby Genes

The Hfr- and F'-mapping data had placed the fhlC mutation between 65 and 69 min in the E. coli chromosome. In order to determine the exact location of fhlC mutation in this region of the chromosome, phage P1-mediated

transduction analysis was performed using appropriate nearby markers.

The fhlC::Tn5 mutant strain (SE1319) as well as the two auxotropic mutant strains (SE1157 and SE1158) carrying the icd mutation and Tn10 (zgg-3::Tn10) near the fhlC gene were used in these experiments. Results from these experiments and co-transduction frequencies of the fhlC::Tn5 mutation with respect to these genes were summarized in Table 10. The icd mutation was co-transduced with fhlC::Tn5 at about 14% and zgg-3::Tn10 at 53 to 59% (Table 10). The zgg-3::Tn10 mutation was co-transduced with fhlC mutation at 43% when strain SE1325 (fhlC::Tn5) was used as a recipient. However, the co-transduction frequency of these two genes was reduced to 29%, when SE1100 (fhlC::lambda placMu) was used as a recipient strain, because of the large size of the lambda placMu DNA insertion in this strain. Therefore, the 43% frequency is closer to the real co-transduction frequency between fhlC and zgg-3::Tn10. Based upon these data, the order of these three genetic markers was established as -icd- zgg-3::Tn10-fhlC::Tn5- (Fig. 6).

In order to determine the chromosomal location of these genes, co-transduction frequencies of the fhlC mutation with respect to metC (65 min) and argG (69 min) genes were sought. The fhlC::Tn5 mutation in SE1319 was transduced into a metC, argG double mutant (AT2699) and $Km^r$ transductants were selected. After several attempts, this particular tranduction experiment produced only a few

Table 10. Transductional analysis of fhlC gene with respect to icd and hup

| Recipient (Genotype) | Donor (Genotype) | Selected Phenotype | # tested | Unselected Phenotype | % |
|---|---|---|---|---|---|
| SE1157 (icd, zgg-3::Tn10) | SE1319 (fhlC::Tn5) | $Icd^+$ | 272 | $Km^r$ | 14 |
| SE1158 (icd, zgg-3::Tn10) | SE1319 (fhlC::Tn5) | $Icd^+$ | 205 | $Km^r$ | 13 |
| SE1100 (fhlC::lac) | SE1157 (icd, zgg-3::Tn10) | $Tc^r$ | 699 | $Icd^-$<br>$Km^s$ | 53<br>29 |
| SE1325 (fhlC::Tn5) | SE1157 (icd, zgg-3::Tn10) | $Tc^r$ | 380 | $Km^s$ | 43 |
| SE29 (hupA) | SE1319 (fhlC::Tn5) | $Hup^+$ | 832 | $Km^r$ | 37 |
| SE42 (hupB) | SE1319 (fhlC::Tn5) | $Hup^+$ | 72 | $Km^r$ | 42 |
| SE31 (hupB) | SE1325 (fhlC::Tn5) | $Hup^+$ | 270 | $Km^r$ | 37 |

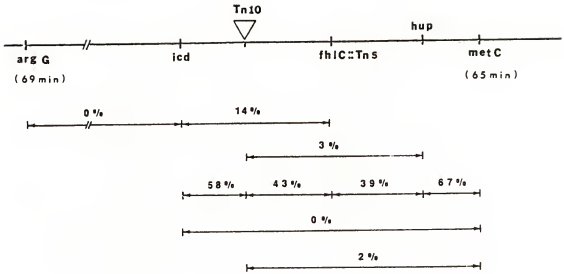



Fig. 6. Genetic map location of the fhlC gene in the E. coli chromosome, based on bacteriophage P1-mediated transductional analysis.

colonies. Transduction of fhlC::Tn5 from SE1319 into another strain carrying metC alone (AT2446) also yielded very few transductants. Thus, as an alternative, co-transduction frequency of fhlC::Tn5 with respect to hupA was determined. The hup genes were reported to code for proteins essential for hydrogen uptake activity and located near metC locus (8). The linkage between the hup genes and fhlC::Tn5 mutation was found to be between 37 and 42% (Table-10).

The icd mutation was found to be unlinked with either metC or argG (Table 11). The co-transduction frequency of metC with the zgg-3::Tn10 mutation in strain SE1157 was between 1 and 3%. However, no linkage was observed between the Tn10 and argG gene.

Linkage between the hup genes and metC gene was determined using two representative strains of each hup locus; SE29 (hupA) and SE31 (hupB). The hupA mutation was co-transduced with metC at 66% and the hupB locus was found to be linked with metC at between 65 and 69% co-transduction frequency (Table 12). These data are consistent with the previous report (62) in that the hup genes are closely linked to metC. The zgg-3::Tn10 in strain SE1157 was co-transduced only at 3% with both hup genes. These results suggest that the fhlC::Tn5 is close to metC, since the cotransduction frequency between this gene and hup gene was about 40% which in turn is 67% co-transduced with metC. Because of the difficulty in obtaining transductants in

Table 11. Transductional analysis of *icd* and *zgg*-3::Tn10 with respect to the *met*C and *arg*G genes

| Recipient (Genotype) | Donor (Genotype) | Selected Phenotype | # tested | Unselected Phenotype | % |
|---|---|---|---|---|---|
| SE1157 (*icd*, *zgg*-3::Tn10) | AT2699 (*met*C, *arg*G) | $Icd^+$ | 66 | $Met^-$ | 0 |
| | | | | $Arg^-$ | 0 |
| | | | | $Tc^s$ | 58 |
| SE1158 (*icd*, *zgg*-3::Tn10) | AT2699 (*met*C, *arg*G) | $Icd^+$ | 87 | $Met^-$ | 0 |
| | | | | $Arg^-$ | 0 |
| | | | | $Tc^s$ | 59 |
| AT2699 (*met*C, *arg*G) | SE1157 (*icd*, *zgg*-3::Tn10) | $Tc^r$ | 475 | $Arg^+$ | 0 |
| | | | | $Met^+$ | 3 |
| AT2447 (*met*C) | SE1157 (*icd*, *zgg*-3::Tn10) | $Tc^r$ $Met^+$ | 601 326 | $Met^+$ $Tc^r$ | 1 1 |

Table 12. Transductional analysis of *hup* with respect to *metC* and *icd*

| Recipient (Genotype) | Donor (Genotype) | Selected Phenotype | # tested | Unselected Phenotype | % |
|---|---|---|---|---|---|
| SE29 (*hupA*) | AT2699 (*metC*, *argG*) | $Hup^+$ | 100 | $Met^-$ | 66 |
| SE31 (*hupB*) | AT2446 (*metC*) | $Hup^+$ | 199 | $Met^-$ | 65 |
| SE31 (*hupB*) | AT2699 (*metC*, *argG*) | $Hup^+$ | 100 | $Met^-$ | 69 |
| SE29 (*hupA*) | SE1157 (*icd*, *zgg*-3::Tn10) | $Hup^+$ | 317 | $Tc^r$ | 3 |
| SE42 (*hupB*) | SE1157 (*icd*, *zgg*-3::Tn10) | $Hup^+$ | 141 | $Tc^r$ | 3 |

experiments involving *fhlC* and *metC* genes it is not clear whether the *fhlC* resides in between *argG* and *metC* genes. However, this question was clearly answered by the Hfr-mediated mating experiment described in a later section. In this experiment an Hfr strain (SE1459) carrying the origin of the transfer in the *fhlC* gene, transferred *argG* gene at high frequency without transfer of *metC* gene during a 15-min mating duration. Based on these results, the gene order of *icd*, *zgg*-3::Tn10, *fhlC* with respect to *metC* and *argG* was established as -*argG*-*icd*-*zgg*-3::Tn10-*fhlC*-*metC*- (Fig. 6). Due to the lack of *fhlC*/*metC* co-transduction frequency data, relative location of the *hup* with respect to *metC* cannot be ascertained.

## Physical Characterization of fhlC Gene

### Cloning the fhlC Gene

Based upon the unique biochemical characteristics and map location, the fhlC gene was established as a hitherto undescribed gene. In order to further characterize this gene in detail, wild type $fhl^+$ DNA was cloned into recombinant plasmids.

To clone $fhlC^+$ gene, strain SE1100 was transformed with the genomic bank (90) containing E. coli chromosomal DNA fragments in plasmid pBR322 as vector. In order to induce FDH2 activity, the $Ap^r$ transformants were selected on LB plus ampicillin agar and incubated in an anaerobic jar, at 37°C, for 18 h. The anaerobically grown $Ap^r$ transformants were tested for the presence of FDH2 activity by pouring 3 ml of soft afar overlay containing FDH2 assay mixture in 0.6% agar. Among several hundred $Ap^r$ transformants tested, one colony which showed rapid formate-dependent BV reduction was isolated. The plasmid present in this clone was isolated and retransformed into strain SE1100. The resulting transformants produced normal levels of FDH2 and FHL activities after anaerobic growth in LB/glucose medium. This plasmid was designated as pSE1001 and maintained for further study.

Subcloning the DNA Coding for fhlC Gene

To examine the location of the fhlC gene in the chromosomal DNA insert of the plasmid pSE1001, its plasmid derivatives which contain various restriction fragments of the chromosomal DNA were constructed.

The size of the plasmid pSE1001 was determined to be 14.3 kb containing a 9.8 kb chromosomal DNA insert (Fig. 7). The chromosomal DNA insert contained three cleavage sites for restriction endonuclease, KpnI, two sites for both PvuII and ClaI, and a single site for EcoRV, XhoI and AvaI (Fig. 7). The vector (pBR322) has one site for PvuII, ClaI, EcoRV, and KpnI (13, 24). Complete digestion of plasmid pSE1001 with enzymes PvuII and ClaI produced six fragments (0.63, 1.32, 1.68, 2.32, 3.57, and 4.80 kb). The 4.8 kb fragment was ligated with 2.32 kb PvuII/ClaI fragment of plasmid vector pBR322. The resulting plasmid (pSE1004) was found to fully complement the fhlC mutation in strain SE1100 (Table 13). The plasmid pSE1001 was again completely digested by KpnI/EcoRV to produce 5 fragments (0.60, 1.20, 2.86, 4.36, and 5.31 kb). The 5.31 kb fragment contained most of the vector DNA and 1.14 kb BamHI/KpnI chromosomal DNA fragment. The 5.31 and 2.86 kb KpnI/EcoRV fragments of pSE1004 were ligated together. The resultant plasmid, pSE1007, was also found to completely complement the fhlC mutation in strain SE1100 (Table 13). Since the 2.86 kb EcoRV/KpnI fragment is the only chromosomal DNA insert present in both plasmid pSE1004 and pSE1007, which

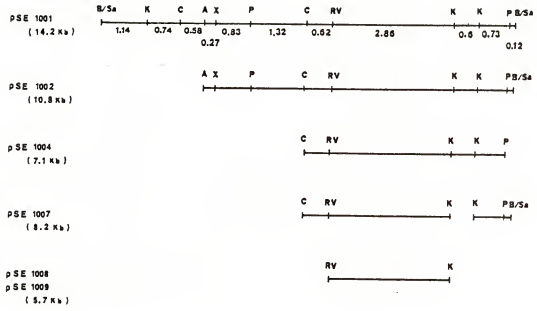



Fig. 7. Restriction endonuclease disgestion map of E. coli chromosomal DNA inserts containing fhlC gene. Plasmid pBR322 was used as vector for all plasmids, except for pSE1008 and pSE1009. Plasmids pUC18 and pUC19 were used as vectors for the construction of pSE1008 and pSE1009, respectively. Abbreviations: A, AvaI; C, ClaI; K, KpnI; P, PstI; RV, EcoRV; X, XhoI; B/Sa, BamHI-Sau3A junction of the vector and chromosomal DNA insert. The numbers indicate the size of the DNA fragment between two restriction sites in kb. The numbers in patenthesis represent the size of the plasmid.

Table 13. FHL activity produced by *E. coli* strain SE1100 carrying plasmid pSE1001 and its derivatives

| Strain | Genotype | Plasmid | FHL spec. act. |
|---|---|---|---|
| BW545 | WT | None | 132 |
| SE1100 | *fhlC* | None | UD |
| | | pSE1001 | 190 |
| | | pSE1004 | 171 |
| | | pSE1007 | 153 |
| | | pSE1008 | 149 |
| | | pSE1009 | 150 |
| | | pSE1004-1 | UD |
| | | pSE1009-1 | UD |
| | | pSE1009-2 | UD |
| | | pSE1009-3 | UD |
| | | pSE1009-4 | UD |
| | | pSE1009-5 | UD |
| | | pSE1009-6 | UD |

* nmol $H_2$ produced / min x (mg cell protein)$^{-1}$

complemented the fhlC mutation to the full extent, the entire fhlC gene appears to reside in this EcoRV/KpnI chromosomal DNA fragment. It was confirmed by further cloning of a 3.Ø2 kb HindIII/KpnI fragment (Ø.16 kb HindII/EcoRV pBR322 sequence plus 2.86 kb EcoRV/KpnI chromosomal DNA fragment) into a HindIII/KpnI-double digested plasmid vector pUC18 and pUC19 (plasmids pSE1ØØ8 and pSE1ØØ9 respectively; Fig. 7). The resultant plasmids, pSE1ØØ8 and pSE1ØØ9, complemented the fhlC mutation and allowed the production of FHL activity to wild type level when introduced into strain SE1100 (Table 13).

Physical Mapping of fhlC Gene

In order to determine the location of the fhlC gene in the 2.86 kb EcoRV/KpnI chromosomal DNA insert more accurately, transposon Tn5 insertion mutations of fhlC gene in plasmid pSE1ØØ9 were selected by following the same method as described in the previous section.

A total of 6 independent plasmids, pSE1ØØ9-1 to pSE1ØØ9-6, which carry transposon Tn5 insertion in the fhlC gene, were identified by checking their phenotype in the fhlC mutant strain SE1187 (Table 13). Transposon Tn5 carries only one cleavage site for the enzymes SalI and BamHI and their position within the transposon is known (56). Since plasmid pSE1ØØ9 has a single cleavage site for enzyme EcoRI and Tn5 does not have restriction enzyme EcoRI cleavage site, the point of insertion and orientation of the

Tn5 insertion in the plasmids can be determined by digestion of each plasmid DNA with EcoRI and SalI, as well as EcoRI and BamHI restriction enconucleases. The results are presented in Fig. 8 and the location and orientation of each transposon Tn5 in the fhlC gene are also summarized. These Tn5 insertions were localized within a Ø.44 kb region of DNA starting from Ø.23 kb from the KpnI cleavage site of the chromosomal insert. Therefore, the fhlC gene resides in a chromosomal DNA region which is closer to the KpnI site.

## Transcriptional Orientation of the fhlC Gene

The orientation of transcription of the fhlC::lambda placMu fusion was determined by a modification of the method developed for determining the orientation of transposon TnlØ insertions (2Ø).

In order to determine the direction of transcription in the fhlC gene, a temperature-sensitive episome $F'_{ts}$ 114 lac zzf::TnlØ (2Ø) was introduced into the fhlC::lambda placMu fusion strain, by conjugation, using S. typhimurium strain TT627 as a donor and E. coli strain SEl438 as a recipient. Ex-conjugants, carrying the F' episome, were selected, at 37°C, on lactose minimal agar containing tetracycline. Several E. coli ex-conjugants were isolated and confirmed by checking their streptomycin sensitivity. One of the ex-conjugants (SEl443) was grown at 42°C on lactose minimal agar to select for the integration of the thermosensitive episome by recombination between the

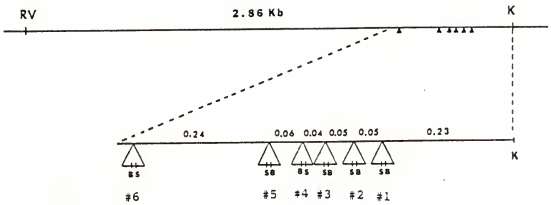



Fig. 8. Physical map location of Tn5 insertions in the fhlC gene of E. coli. Abbreviations: B, BamHI; K, KpnI; RV, EcoRV; S, SalI. The numbers indicate the size of DNA fragment in Kb, either between two Tn5 insertion sites or between a Tn5 insertion site and a restriction site. These Tn5 locations represent the fhlC::Tn5 mutations in plasmids pSE1009-1 to 6 which are represented as #1 through 6.

fhlC::lambda placMu fusion in the chromosome and homologous lac DNA sequence present in the episome. One of the resultant recombinant Hfr strains, SE1459, was used in the conjugation experiments. The direction of transfer of chromosomal DNA by the Hfr strain will be dependent on the orientation of the lac DNA in the chromosome (Fig. 9). The direction of transcription of the fhlC gene can be determined from the orientation of the lac DNA, because in the original fhlC::lambda placMu fusion (SE1438), lac DNA is positioned downstream from the promoter of fhlC gene. Chromosomal transfer by the Hfr strain occurs only in one direction from the site of integration. Since the orientation of the lac operon relative to oriT in the $F'_{ts}$ 114 lac zzf::Tn10 is known, only the genes that are distal to the fhlC promoter are transferred early (Fig. 9). Since the fhlC::lac fusion mapped at 66 min in E. coli chromosome, as described in the previous section, argG (69 min) and metC (65 min) were chosen as possible nearby genes for this experiment. The Hfr strain, SE1443, was conjugated with strain AT2699 (metC, argG) for 15 min and $Arg^+$ or $Met^+$ recombinants were selected. Only the argG gene, located clockwise from fhlC gene, in the genetic map (8) was transferred at high frequency. The $metC^+$ gene, which lies counter-clockwise from the fhlC gene, was not transferred during the 15-min mating period (Table 14). These results clearly show that the direction of transcription of the fhlC gene is clockwise along the E. coli chromosome.

Transcriptional Orientation of fhlC Gene

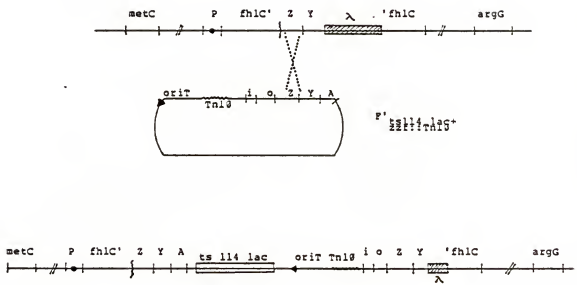



Fig. 9. Diagrammatic representation of the construction of an Hfr strain using the lac homology in fhlC gene. Integration of the F' $_{ts}$114 lac$^{+}$ zzf::Tnlø episome in the strain SE1459 chromosome occurs via recombination between the homologous lac sequence present in the fhlC gene (insertional mutagenesis) in the chromosome and F' element. Orientation of nearby genes as a result of this integration is presented. Genes which are distal to the fhlC promoter will be transferred early, by the Hfr, to appropriate recipient strains.

Table 14. Transcriptional orientation of the *fhlC* gene measured as direction of transfer of chromosomal genes

| Recipient | Donor | # of recombinants/ $10^8$ donor cells | |
|---|---|---|---|
| | | $Arg^+$ | $Met^+$ |
| AT2699 (*metC*, *argG*) | SE1443 (Hfr) | 769 | Ø |

## Regulation of/by the fhlC Gene

### Regulation of fhlC Gene Expression

The existance of a lacZ fusion in a gene of interest allows a detailed analysis of factors regulating the target gene (96). Using the fhlC::lac fusion strain (SE1100), the effect of physiological conditions and other genes on the expression of the fhlC gene can be easily monitored by measuring beta-galactosidase activity.

It is known that formate hydrogenlyase activity is repressed by oxygen and nitrate (28, 106). In order to test the effect of these terminal electron acceptors on fhlC gene expression, beta-galactosidase activity, expressed from fhlC gene promoter, was determined in the presence or absence of these compounds. As can be seen from the data presented in Table 15, the levels of beta-galactosidase activity, produced by strain SE1100, was not influenced by the presence of either oxygen or nitrate in the growth medium.

Recently, we have demonstrated that a mutant strain, defective in endogenous formate production (pfl), due to a defect in pyruvate formatelyase, failed to produce both formate hydrogenlyase and formate dehydrogenase 2 as well as FHL-hydrogenase activity (unpublished data). These activities were restored by addition of formate exogenously to the growth medium (unpublished data). These findings show that formate is required as an inducer for the production of formate hydrogenlyase activity. The effect of

Table 15. Regulation of transcription of fhlC gene in different genetic backgrounds

| Strain (relevant-genotype) | Medium | Supplement* | Beta-galactosidase** $+O_2$ | Beta-galactosidase** $-O_2$ |
|---|---|---|---|---|
| SE1100 (fhlC::lac) | LB/Glc | None | 212 | 236 |
| | | + Mo | 212 | 258 |
| | | + $NO_3^-$ | 226 | 228 |
| | | + cAMP | 226 | 229 |
| SE1194 (fhlC::lac, cya) | LB/Glc | None | 237 | 233 |
| | | + cAMP | 246 | 235 |
| SE1226 (fhlC::lac, fnr) | LB/Glc | None | 213 | 257 |
| SE1308 (fhlC::lac, pfl) | LB | None | 216 | 336 |
| | | + formate | 218 | 321 |

* 1 mM Mo, 20 mM $NO_3^-$, 5 mM cAMP, or 0.1% formate was added in medium as indicated.

** Beta-galactosidase activity is expressed as nmol x $(min)^{-1}$ x $(mg\ cell\ protein)^{-1}$.

formate on the fhlC expression was examined by constructing an fhlC::lac, pfl double mutant strain, SE1308. Introduction of the pfl mutation into the strain SE1100 produced no difference in aerobic expression of fhlC gene. However, under anaerobic conditions, the expression was enhanced slightly both with and without formate in the medium (about 50% increase over the aerobic expression level).

Cyclic AMP (cAMP) was reported to enhance formate hydrogenlyase activity (82). In this laboratory, it has been shown that active adenylate cyclase, which catalyzes the production of cAMP, is required for the synthesis and production of formate dehydrogenase 2 and FHL-hydrogenase activity when intracellular formate concentration is low (unpublished data). In order to test whether the effect of cAMP on formate hydrogenlyase induction is through the regulation of fhlC gene expression, cya, fhlC::lac double mutant strains were constructed. One of these cya, fhlC::lac double mutant strains, SE1194, was tested for the level of beta-galactosidase. Again, no difference was observed in the pattern of beta-galactosidase production as compared with that of the $cya^+$ parent strain (SE1100). Addition of cAMP to the growth medium did not make any difference in either strain.

Mutant strains which have a defect in a pleiotropic anaerobic regulatory gene (fnr) which has been postulated to regulate the synthesis of fumarate reductase and nitrate

reductase have been reported to be partially deficient in formate hydrogenlyase activity (60). It is possible that the effect of the fnr gene product on FHL activity may be mediated through the fhlC gene product. The effect of fnr gene on the fhlC gene expression was studied by constructing a fnr, fhlC::lac double mutant strain, SE1226. As presented in Table 15, the introduction of fnr mutation in fhlC::lac fusion strain had no effect on the expression of fhlC gene under either aerobic or anaerobic growth conditions. This result clearly shows that the fnr-mediated control mechanism on the formate hydrogenlyase is different from that of fhlC mediated one.

Since the addition of molybdate to the growth medium restored the deficiency in enzyme activities in the fhlC mutant strain (SE1100) (Table 5), the effect of molybdate on the expression of fhlC gene was examined in the presence and absence of molybdate supplement in the medium. The presence of molybdate did not change the levels of beta-galactosidase activity in cells grown under either aerobic or anaerobic condition (Table 15).

## Effect of the fhlC Mutation on the Biosynthesis of Formate Hydrogenlyase

Presence of a mutation in the fhlC gene abolished the activities of formate dehydrogenase 2 and FHL-hydrogenase, both of which are required for formate hydrogenlyase activity (Table 2 and 4). In order to understand the role

of fhlC gene in the regulation of formate hydrogenlyase, the effect of fhlC mutation on the expression of genes essential for formate hydrogenlyase was examined using lac fusion strains, where the structural gene for beta-galactosidase was fused to the promoter of the three different genes essential for formate hydrogenlyase activity.

Role of fhlC Gene on the Expression of fdhF::lac

Strain M9s carries a lac fusion in the structural gene for formate dehydrogenase 2 (fdhF::lac) which encodes an 80 kd selenopeptide (83). The role of the fhlC gene in the expression of the FDH2 structural gene was tested by constructing an fhlC, fdhF::lac double mutant strain. A mutational allele of fhlC was transduced into strain M9s using strain SE1319 (fhlC::Tn5) as the donor. The kanamycin-resistant transductant was confirmed for fhlC phenotype by checking for the absence of formate dehydrogenase 1 activity which is also absent in fhlC mutants (Table 1). All the $Km^r$ transductants (a total of 10 strains tested) lacked formate dehydrogenase 1 activity. One of the resulting double mutants, strain SE1334 (fdhF::lac, fhlC::Tn5) was tested for beta-galactosidase activity (Table 16). In the fdhF parent strain (M9s), the expression of beta-galactosidase activity was near zero levels under aerobic conditions (4U), but the enzyme was derepressed under anaerobic conditions (342U) which is in

Table 16. Molybdate-dependent expression of *fdhF*, *hyd*, and *ant* gene in *fhlC* genetic background

| Strain | Relevant genotype | $O_2$ * | Beta-galactosidase ** | | |
|---|---|---|---|---|---|
| | | | - | +Mo | +W |
| M9s | *fdhF::lac* | + | 4 | 4 | 3 |
| | | - | 342 | 428 | 423 |
| SE1334 | *fdhF::lac* *fhlC::Tn5* | + | 2 | 2 | 3 |
| | | - | 24 | 448 | 368 |
| M3s | *hyd::lac* | + | 8 | 8 | 2 |
| | | - | 663 | 696 | 893 |
| SE1328 | *hyd::lac* *fhlC::Tn5* | + | 2 | 1 | 2 |
| | | - | 3 | 674 | 599 |
| GW5310 | *ant::lac* | + | 1 | 1 | 1 |
| | | - | 854 | 808 | 966 |
| SE1473 | *ant::lac* *fhlC::Tn5* | + | 1 | 1 | 1 |
| | | - | 62 | 811 | 553 |

* Cells were cultured under aerobic or anaerobic conditions as decribed in "Materials and Methods" section.

** Beta-galactosidase activity is expressed as nmol x $(min)^{-1}$ x $(mg\ cell\ protein)^{-1}$.

Molybdate and tungstate were added to LB/glucose (0.3%) medium at a final concentration of 1 mM.

agreement with the reported values (84). However, the anaerobic derepression of fdhF gene was decreased to about 7% of fhlC$^{+}$ wild type levels, in the double mutant. These results clearly show that the active gene product of the fhlC gene is required for the transcription of the formate dehydrogenase 2 gene.

The deficiency of formate dehydrogenase 2 activity in the fhlC mutant strain was completely restored to wild type phenotype by addition of molybdate to the growth medium (Table 4 and Fig. 1, 2, 3). In the parent strain (M9s), the presence of molybdate did not result in any significant qualitative difference in the beta-galactosidase levels, although presence of molybdate increased the expression by 25% under anaerobic conditions. However, in the fhlC, fdhF double mutant (SE1334), molybdate at a concentration of 1 mM increased the beta-galactosidase activity to the parent level (from 24U to 448U). These results show that molybdenum is an essential requirement for the transcription of the formate dehydrogenase 2 gene.

Tungstate is known as a competitive inhibitor of molybdate (65), and growth of E. coli in media containing tungstate leads to accumulation of inactive molybdoenzymes (34, 100). It was of interest to test whether addition of tungstate affected the expression of fdhF::lac fusion. Strains M9s and SE1337 were grown in LB/Glucose (0.3%) medium, both in the presence and absence of tungstate (1 mM) supplement and the levels of beta-galactosidase activity

were determined (Table 16). In anaerobically-grown strain SE1334, the presence of tungstate in the medium completely derepressed the fdhF::lac expression to the level of the wild type, grown without tungstate supplement. In strain M9s, addition of tungstate slightly increased beta-galactosidase level by 25%. These results show that the presence of tungstate also allows derepression of the formate dehydrogenase 2 gene and the extent of derepression by molybdate and tungstate are very similar.

Role of fhlC Gene on the Expression of hyd::lac

Hydrogenase activity associated with formate hydrogenlyase activity (FHL-hydrogenase) was also absent in the fhlC mutant, strain SE1100 (Table 6). The same mutation abolished formate dehyrogenase 2 activity and its effect was found to be at the transcriptional level of the structural gene of the enzyme. Thus, it was of interest to test whether the effect of the fhlC mutation on the FHL-hydrogenase is also at the transcriptional level.

A lac fusion strain, M3s (hyd::lac), carries a lac fusion in a hydrogenase gene which is reported to be essential for FHL-hydrogenase (111). The effect of fhlC mutation on the expression of the hyd::lac was examined by constructing an fhlC, hyd::lac double mutant strain. Strain SE1319 (fhlC::Tn5) was used as a donor strain for transduction of the fhlC mutation into strain M3s (hyd::lac). Kanamycin-resistant transductants were

confirmed to carry the fhlC::Tn5 mutation by checking for the absence of formate dehydrogenase 2 activity, after growth in LB medium. One of the representative double mutant strains, SE1328 (hyd::lac, fhlC::Tn5), and its parent strain were tested for the expression of hyd::lac after growth in various media under different growth conditions (Table 16). The expression of the hyd gene was completely repressed by oxygen (8U), but derepressed by anaerobiosis (663U) which is consistent with the previous report that this gene is derepressed only under anaerobic growth conditions. Introduction of the fhlC mutation into this hyd::lac fusion strain completely abolished the anaerobic expression of the hyd gene (from 663U to 3U). Thus, it is clear that the active fhlC gene product is also required for the transcriptional expression of the hyd gene.

The mutational effect of fhlC on the expression of hyd::lac was also suppressed by the addition of either molybdate or tungstate in the growth medium as in the case of fdhF::lac fusion.

Role of fhlC Gene on the Expression of ant::lac

A mutant strain carrying a mutation in fhlC gene was deficient in both FDH2 and FHL-hydrogenase activities both of which are components of FHL complex (Table 2 and Table 4). The deficiency of these enzyme activities in the mutant was found to be due to the lack of transcriptional expression of the two independent genes essential for these

enzyme activities (Table 16). These pleiotropic effects of the fhlC mutation on the synthesis of these enzymes suggest that production of other components (electron carriers) involved in the same metabolic pathway may also be regulated in the same manner as a regulon.

This possibility was tested by examining the effect of the fhlC mutation on the expression of a gene (ant) reported to be essential for the production of an anaerobic electron transport protein involved in the FHL pathway (110). A mutational allele of fhlC gene (fhl::Tn5) was transduced from strain SE1319 into strain GW5310 which carries a lac fusion in the ant gene (ant::lac). Kanamycin-resistant transductants were tested to confirm the fhlC phenotype by checking for lack of FDH1 activity. One of the fhlC::Tn5, ant::lac double mutants, strain SE1473, was tested for the expression (as beta-galactosidate activity) after growth in various growth conditions (Table 16). Beta-galactosidase levels in the parent ant::lac strain (GW5310) were strongly derepressed in cultures grown anaerobically (854U). The high-level ant::lac expression during anaerobic growth was completely repressed during aerobic growth (1U). These results are in agreement with the previous report of anaerobic expression of ant gene (110). However, the high-level anaerobic expression of the ant gene was severely repressed by introduction of fhlC::Tn5 mutation into the ant::lac strain (from 854U to 62U). These results clearly

show that the active fhlC gene product is also required for the transcriptional expression of ant gene.

Addition of molybdate or tungstate in the growth medium also suppressed the mutational effect of fhl on ant::lac expression as in the cases of fdhF::lac and hyd::lac.

These results clearly demonstrate that the three different genes (fdhF, ant, hyd) essential for the production of three components of formate hydrogenlyase pathway are coordinately regulated at transcriptional level.

## DISCUSSION

*E. coli* is capable of utilizing several different terminal electron acceptors during growth (41). These include $O_2$, $NO_3^-$, and organic compounds, etc. The end products of glucose metabolism depend on the availability of these compounds. The major end products of glucose catabolism, under fermentative conditions, are acetate, lactate, ethanol, $H_2$ and $CO_2$. The production of the gaseous products, $H_2$ and $CO_2$, from formate is catalyzed by a membrane-bound multienzyme complex, formate hydrogenlyase, which is composed of formate dehydrogenase 2 and a hydrogenase (FHL-hydrogenase), linked by an unknown number of unidentified intermediate electron carriers. A number of different genes, necessary for the activity of this unique biochemical pathway (FHL), have been identified. However, their regulation is poorly understood. The formate hydrogenlyase system is especially hard to study because of the inherent difficulty of working with proteins that are $O_2$ sensitive, and with electron transport proteins with no enzymatic activity.

In order to understand the regulation of the formate hydrogenlyase pathway, four independent *lac* fusion strains defective in formate dehydrogenase activity were isolated.

An operon fusion-generating lambda phage, lambda placMu 53, was used as a means of mutagenesis and a dye-overlay procedure (71) was used as an identification method in the experiment. Among the four mutants isolated, the mutant strain SE1100 is deficient in formate dehydrogenase activities (both FDH1 and FDH2) and FHL-hydrogenase activity (Table 2 and Table 4). Therefore, the absence of FHL activity observed in this mutant strain results from the lack of both FDH2 and FHL-hydrogenase activities. The effect of the mutation present in strain SE1100 on these enzyme activities was reversed by addition of molybdate to the growth medium (Table 5, Fig. 1, 2, and 3). The deficiency of FDH1 activity in the mutant strain was also overcome by molybdate in the growth medium, since this strain grew well in glycerol/$NO_3^-$ medium which contained molybdate at a concentration of 46 uM.

It has been reported that mutant strains carrying mutations in chlD gene, mapping at 17 min in the chromosome, lacked FHL and FDH1 activities as well as nitrate reductase acitivity (63, 105) and the effect of the chlD mutation was also reversed by addition of high concentrations of molybdate (1 mM) to the growth medium. Although the phenotype of strain SE1100 is similar to that of chlD mutants, genetic analysis located the altered gene in this mutant at 66 min in the E. coli chromosome (Table 10, 11, 12 and Fig. 6). This difference in the genetic map location distinguishes the altered gene in strain SE1100 from the

chlD gene. Since no mutants with any of these properties have been described near 66 min of the E. coli chromosome, it is concluded that the mutation in strain SE1100 defines a hitherto unidentified gene. Since the presence of mutation in this locus abolished formate hydrogenlyase activity (due to the the pleiotropic defects in at least two components of formate hydrogenlyase pathway: FDH2 and FHL-hydrogenase), the mutational locus was designated as fhlC, meaning defective in FHL activity, following after previously identified fhlA gene (90) near hydB (90) and fhlB (unpublished data) near hydA (90) .

The wild type allele of the fhlC gene was cloned in a recombinant plasmid (pSE1001) and found to reside within a 2.86 kb EcoRI-KpnI fragment of E. coli chromosomal DNA (Fig. 6). The physical mapping of the fhlC gene by transposon Tn5 mutagenesis showed that the DNA coding for the fhlC gene resides closer to the KpnI cleavage site than to the EcoRV site in the chromosomal DNA (Fig. 7). The transcriptional orientation of the fhlC gene was found to be clockwise along the E. coli chromosome. Wild type $fhlC^+$ gene was dominant to fhlC mutational allele (Table 13).

Formate dehydrogenase 1 has been purified and shown to be a molybdoenzyme (31). Although formate dehydrogenase 2 has not been purified, based upon physiological and genetic data (34, 63, 105), it is generally believed to be a molybdo-protein also. Therefore, it is not surprising that a mutation affecting the cell's utilization of molybdate has

pleiotropic effects on both molybdoenzymes. The activities of other molybdoenzymes in strain SE1100 were not tested. However, it is possible that this mutant also lacks these enzymes in the absence of molybdate supplement in the growth medium. The deficiency in the production of FHL-hydrogenase activity in this strain is not easily explainable, because of the lack of physiological and biochemical information on this enzyme or pathway. A possible explanation would be that the deficiency of the FHL-hydrogenase activity in the fhlC mutant is a physiological consequence of failure in formate dehydrogenase 2 enzyme production. It is possible that when a cell fails to produce active formate dehydrogenase 2, a component of FHL complex, other components (including FHL-hydrogenase and electron transport proteins) in the same metabolic pathway also may not be produced. This interpretation is supported by the observation that the three different genes (fdhF, ant, hyd) which are essential for the production of the three components of the formate hydrogenlyase pathway are also coordinately regulated at transcriptional level by the fhlC gene product (Table 16). An alternative explanation could be that the FHL-hydrogenase is also a molybdoenzyme, sharing a common molybdo-cofactor with other molybdoenzymes. However, among many hydrogenase proteins purified from various bacterial sources, including the two hydrogenase isoenzymes isolated from E. coli, none were reported to

contain molybdenum as a cofactor (3, 10, 92); thus, the second possibility is unlikely.

Since the fhlC mutation showed pleiotropic effects on the molydboenzymes (FDH1 and FDH2), it is possible that the active fhlC gene product is involved in the utilization of molybdate, either its transport into the cells, synthesis of molybdo-cofactor, or its conversion to the proper redox state. The observation that the addition of high concentrations of molybdate to the growth medium reversed the pleiotropic effect of fhlC mutation suggests that the participation of fhlC gene product in molybdate transport is most likely.

The inhibitory effect of selenium on the production of molybdate-dependent FHL activity in the fhlC mutant also suggests that the fhlC gene product is involved in molybdate transport. It is possible that at high concentrations, molybdate is transported through a so far unidentified selenium-transport system which has a much lower efficiency for molybdate transport. Thus, at relatively high concentrations, selenite probably competes against molybdate and excludes the transport of molybdate.

Tungstate, an analog of molybdenum, is known to be incorporated into molybdenum-deficient apomolybdoproteins when the cells were grown in molybdenum-deficient medium (34, 100). Addition of tungstate to the growth medium also reversed the pleiotropic effect of the fhlC mutation on the transcription of the three genes essential for FHL complex

(Table 16). Since these metal ions have very similar chemical properties (5), molybdate and tungstate are probably transported into the cell via a common transport system (17, 30, 77).

Based upon these results, it is concluded that the fhlC gene product is involved in molybdate transport. Another gene whose gene product was proposed to be involved in molybdate transport is chlD gene (51). Recently, Miller et al. (73) examined the regulation of the chlD gene, using chlD::lac fusion strains. These investigators reported that the chlD gene was expressed when cells were grown with less than 10 nM molybdate and was completely repressed at molybdate concentrations higher than 500 nM in the growth medium, under both aerobic and anaerobic conditions. Addition of nitrate to the growth medium stimulated the chlD expression two- to three-fold, under anaerobic conditions. However, the expression of the fhlC gene was not influenced by either nitrate or molybdate addition to the medium under either aerobic or anaerobic conditions (Table 15). Therefore, although the fhlC and chlD mutants showed similar phenotypes, the roles of these genes in the molybdate utilization in the cell seem to be different.

The expression of the fhlC gene is also independent of $O_2$ or formate and also is not influenced by either the presence of cya or fnr gene product (Table 15). On the other hand, the expression of the three genes involved in FHL (fdhF, ant and hyd) are induced only under anaerobic

growth conditions and in the absence of an inorganic electron acceptor, $NO_3^-$ (84, 110). The expression of fdh::lac and hyd::lac genes are specifically enhanced by the presence of formate in anaerobic growth medium (84). In an fhlC background, molybdate is needed for the transcription of the fdhF, ant and hyd genes (Table 16). The $fhlC^+$ wild type strain scavenges this metal ion from the growth medium and supports the full expression of these genes even without supplementation of molybdate to the growth medium (Table 16). These differences show that the molybdate-mediated regulation of FHL production by fhlC gene is independent of the other control systems operating on this pathway.

The role of molybdenum in regulating the synthesis of molyboenzymes has been controversial. This has led to a number of studies on the deprivation of molybdate on molybdoenzymes in various organisms, revealing no uniform response (27, 40, 47, 50). In E. coli the effect of molybdate on the expression of the structural gene for nitrate reductase was examined using nar::lac fusion strains by Pascal and her coworkers (78). These investigators showed that the transcription of the nitrate reductase gene is stimulated by molybdenum in the chlD mutant background. However, in chlD, nar::lac fusion strains the expression of the nar gene was up to 30% of the maximal level of the $chlD^+$ parental strain when nitrate was added to the growth medium, even without molybdate. These results suggest that the nitrate reductase gene can still be induced in the chlD

mutant strain at a substantial level even without molybdate addition to the growth medium. Thus, it would be interesting to test the effect of molybdate on the expression of the nar::lac fusion in the fhlC mutant background to examine the role of molybdenum in regulating expression of other molybdoenzymes.

The amount of molybdate required for the production of FHL activity by the fhlC mutant was greatly influenced by growth medium (Fig. 1, 2 and 3). Cultures grown in glucose-minimal medium required 50-fold less molybdate than LB plus glucose grown cultures. The requirement for higher concentrations of molybdate in the LB medium suggests that the presence of higher levels of N-compounds, as in the case of LB medium, inhibits the production of the secondary molybdate-transport system. On the other hand, the difference in molybdate concentrations required to reverse the fhlC mutational effect on FHL activity in the cells grown in glucose-minimal and LB plus glucose media could result from the different degrees of production of a highly efficient molybdate transport system in the mutant strain. These interpretations are speculative, but should lead to future experiments to elucidat the mechanism of the "LB-effect".

In summary, a new gene (fhlC) essential for the synthesis and activity of formate hydrogenlyase in E. coli was identified in this study. These findings suggest that three independent genes essential for the three different

components in the formate hydrogenlyase pathway were coordinately regulated at transcriptional level. Although the physiological role of the fhlC gene is believed to be involved in molybdate transport or utilization of molybdate by the cell, addtional experiments are necessary to establish the exact role of the gene product.

REFERENCES


1. Ackrell, B. A. C., R. N. Asato, and H. F. Mower. 1966. Multiple forms of bacterial hydrogenases. J. Bacteriol. 92:828-838.

2. Adams, M. W. W., and D. O. Hall. 1979. Purification of the membrane-bound hydrogenase of Escherichia coli. Biochem. J. 183:11-22.

3. Adams, M. W. W., L. E. Mortenson, and J. -S. Chen. 1981. Hydrogenase. Biochim. Biophy. Acta 594:105-176.

4. Anand, S. R., and A. I. Krasna. 1965. Catalysis of the $H_2$-HTO exchange by hydrogenase. A new assay for hydrogenase. Biochemistry 4:2747-2753.

5. Andreesen J. R. 1979. Role of selenium, molybdenum and tungsten in anaerobes, p.31-40. In G. Gottschalk, N. Pfennig, and H. Werner (ed.), Anaerobes and anaerobic infections. Gustav Fischer-Verlag, Stuttgart.

6. Azoulay, E., and B. Marty. 1970. Etude du systeme multienzymatique hydrogen lyase chez Escherichia coli K12 et ses mutants chlorate-resistants. Eur. J. Bacteriol. 13:168-173.

7. Bachmann, B. J. 1972. Pedigrees of some mutant strains of Escherichia coli K-12. Bacteriol. Rev.36:525-557.

8. Bachmann, B. J. 1983. Linkage map of Escherichia coli K-12, edition 7. Microbiol. Rev. 47:180-230.

9. Ballantine, S. P., and D. H. Boxer. 1985. Nickel-containing hydrogenase isoenzymes from anaerobically grown Escherichia coli K-12. J. Bacteriol. 163:454-459.

10. Ballantine, S. P., and D. H. Boxer. 1986. Isolation and characterization of a soluble active fragment of hydrogenase isoenzyme 2 from the membranes of anaerobically grown Escherichia coli. Eur. J. Biochem. 156:277-284.

11. Berman, M. L., and J. Beckwith. 1979. Fusions of the lac operon to the transfer RNA gene tyrT of Escherichia coli. J. Mol. Biol. 130:285-301.

12. Bernhard, T., and G. Gottschalk. 1978. Cell yields of Escherichia coli during anaerobic growth on fumarate and molecular hydrogen. Arch. Microbiol. 116:235-238.

13. Bolivar, F., R. L. Rodriquez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. L. Falkow. 1977. Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene 2:95-113.

14. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

15. Bremer, E., T. J. Silhavy, J. M. Weisemann, and G. M. Weinstock. 1984. Labmda placMu: a transposable derivative of bacteriophage lambda for creating lacZ protein fusions in a single step. J. Bacteriol. 158: 1084-1093.

16. Brickman, E., L. Soll, and J. Beckwith. 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in Escherichia coli, including deletions of the gene for adenylate cyclase. J. Bacteriol. 116:582-587.

17. Cardin, C. J., and J. Mason. 1976. Molybdate and tungstate transfer by rat ileum. Competitive inhibition by sulphate. Biochim. Biophys. Acta 455:937-946.

18. Casadaban, M. J. 1976. Transposition and fusion of the lac genes to selected promoters in Escherichia coli using bacteriophage lambda and Mu. J. Mol. Biol. 104:541-556.

19. Casse, F. 1970. Mapping of the gene chl-B controlling membrane bound nitrate reductase and formic hydrogen-lyase activities in Escherichia coli K12. Biochem. Biophys. Res. Commun. 39:429-436.

20. Chumley, F. G., R. Menzel, and R. Roth. 1979. Hfr formation directed by Tn10. Genetics 91:639-655.

21. Cole, J.A. 1976. Microbial gas metabolism. Adv. Micro. Physiol. 14:1-87.

22. Cole, J. A., and J. W. T. Wimpenny. 1966. The interrelationships of low redox potential cytochrome $c_{552}$ and hydrogenase in facultative anaerobes. Biochim. Biophys. Acta 128:419-425. Biochim. Biophys. Acta 128:419-425.

23. Cox, J. C., E. S. Edwards, and J. A. DeMoss. 1981. Resolution of distinct selenium-containing formate dehydrogenase from Escherichia coli. J. Bacteriol. 145:1317-1324.

24. Davis, R. W., D. Botstein, and J. R. Roth. 1980. A manual for genetic engineering. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

25. del Campillo-Campbell, A., and A. Campbell. 1982. Molybdenum cofactor requirement for biotin sulfoxide reduction in Escherichia coli. J. Bacteriol. 149: 469-478.

26. DeLucia, P., and J. Cairns. 1969. Isolation of an E. coli strain with a mutation affecting DNA polymerase. Nature 224::1164-1168.

27. Dixon, R., R. R. Eady, G. Espin, S. Hill, M. Iaccarino, D. Kahn, and M. Merrick. 1980. Analysis of regulation of Klebsiella pneumoniae nitrogen fixation (nif) gene cluster with gene fusions. Nature (London) 286: 128-132.

28. Dobrogosz, W. J. 1966. Altered end-product patterns and catabolite repression in Escherichia coli. J. Bacteriol. 91:2263-2269.

29. Drews, G. 1965. Untersuchungen zur Regulation der Bacteriochlorophyll-Synthase bei Rhodospirillum rubrum. Arch. Mikrobiol. 51:185-198.

30. Elliot, B. B., and L. E. Mortenson. 1975. Transport of molybdate by Clostridium pasteurianum. J. Bacteriol. 124:1295-1301.

31. Enoch, H. G. and R. L. Lester. 1975. The purification and properties of formate dehydrogenase and nitrate reductase from Escherichia coli. J. Biol. Chem. 250:6693-6705.

32. Foster, T. J., V. Lundbald, S. Hanley-Way, S. M. Halling, and N. Kleckner. 1981. Three Tn10-associated excision events: relationship to transposition and role of direct and inverted repeats. Cell 23:215-227.

33. Giordano, G., L. Grillett, J. Pommier, C. Terriere, B.A. Haddock, and E. Azoulay. 1980. Precursor forms of the subunits of nitrate reductase in chlA and chlB mutants of Escherichia coli K12. Eur. J. Biochem. 105:297-306.

34. Giordano, G., M. Violet, C. -L. Medani, and J. Pommier. 1984. A common pathway for the activation of several molybdoenzymes in Escherichia coli K12. Biochim. Biophys. Acta 798:216-225.

35. Glaser, J. H., and J. A. DeMoss. 1971. Phenotypic restoration by molybdate of nitrate reductase activity in chlD mutants of Escherichia coli. J. Bacteriol. 108:854-860.

36. Graham, A., H. E. Jenkins, N. H. Smith, M. -A. Mandrand-Berthelot, B. A. Haddock, and D. H. Boxer. 1980. The synthesis of formate dehydrogenase and nitrate reductase proteins in various fdh and chl mutants of Escherichia coli. FEMS Microbiol. Lett. 7:145-151.

37. Gray, C. T., and H. Gest. 1965. Biological formation of molecular hydrogen. Science 148:186-192.

38. Gray, C. T., J. W. T. Wimpenny, D. E. Hughes, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. I. Structural and functional changes in Escherichia coli associated with shifts. Biochim. Biophys. Acta 117:22-32.

39. Gutterson, N. I., and D. E. Koshland, Jr. 1983. Replacement and amplification of bacterial genes with sequences altered in vitro. Proc. Natl. Acad. Sci. 80:4894-4898.

40. Haallenbeck, P. C., and J. R. Benemann. 1980. Effect of molybdenum starvation and tungsten on the synthesis and activity of nitrogenase in Anabaena cylindrica. FEMS Microbiol. Lett. 9:121-124.

41. Haddock, B. A., and C. W. Jones. 1977. Bacterial respiration. Bacteriol. Rev. 41:47-99.

42. Haddodk, B. A., and M. A. Mandrand-Berthelot. 1982. Escherichia coli formate-to-nitrate respiratory chain: genetic analysis. Biochem. Soc. Trans. 10:478-480.

43. Hewitt, E. J., and B. A. Notton. 1980. Nitrate reductase systems in eukaryotic and prokaryotic organisms, p.273-326. In M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.

44. Hill, C. W., and B. W. Harnish. 1981. Inversions between ribosomal RNA genes of Escherichia coli Proc. Natl. Acad. Sci. 78:7069-7072.

45. Hom, S. S. M., H. Hennecke, and K. T. Shanmugam. 1980. Regulation of nitrogenase biosynthesis in Klebsiella pneumoniae: effect of nitrate. J. Gen. Microbiol. 117:169-179.

46. Hong, J. -S. and B. N. Ames. 1971. Localized mutagenesis of any specific small region of the bacterial chromosome. Proc. Nat. Acad. Sci. 68:3158-3162.

47. Imperial, J., R. A. Ugalde, V. K. Shah, and W. J. Brill. 1984. Role of the nifQ gene product in the incorporation of molybdenum into nitrogenase in Klebsiella pneumoniae. J. Bacteriol. 158:187-194.

48. Ingledew, W. J., and R. K. Poole. 1984. The respiratory chains of Escherichia coli. Microbiol. Rev. 48:222-271.

49. Itoh, T., and J. Tomizawa. 1979. Initiation of replication of plasmid ColE 1 DNA by RNA polymerase, ribonuclease H, and DNA polymerase I. Cold Spring Harbor Symposia on Quantitative Biology 53:409-417.

50. Jacobson, M. R., R. Premakumar, and P. E. Bishop. 1986. Transcriptional regulation of nitrogen fixation by molybdenum in Azotobacter vinelandii. J. Bacteriol. 167:480-486.

51. Johann, S., and S. M. Hinton. 1987. Cloning and nucleotide sequence of the chlD locus. J. Bacteriol. 169:1911-1916.

52. Johnson, J. L. 1980. The molybdenum cofactor common to nitrate reductase, xanthine dehydrogenase and suphite oxidae, p. 345-383. In M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.

53. Johnson, J. L., B. E. Hainline, K. V. Rajagopalan, and B. H. Arison. 1984. The pterin component of the molybdenum cofactor: structural characterization of two fluorescent derivatives. J. Biol. Chem. 259:5414-5422.

54. Johnson, J. L., and K. V. Rajagopalan. 1982. Structural and metabolic relationship between the molybdenum cofactor and urothione. Proc. Natl. Acad. Sci. 79:6856-6860.

55. Jones, R. W. 1980. The role of membrane-bound hydrogenase in the energy-conserving oxidation of molecular hydrogen by Escherichia coli. Biochem. J. 188:345-350.

56. Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. 177:65-72.

57. Komeda, Y., and T. Iino. 1979. Regulation of expression of the flagellin gene (hag) in Escherichia coli K-12: analysis of hag-lac gene fusions. J. Bacteriol. 139:721-729.

58. Krasna, A. 1980. Regulation of hydrogenase activity in enterobacteria. J. Bacteriol. 144:1094-1097.

59. Kumamoto, C. A., and J. Beckwith. 1985. Evidence for specificity at an early step in protein export in Escherichia coli. J. Bacteriol. 163:267-274.

60. Lambden, P. R., and J. R. Guest. 1976. Mutants of Escherichia coli K12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. 97:145-160.

61. Leathers, T. D., J. Noti, and H. E. Umbarger. 1979. Physical characterization of ilv-lac fusions. J. Bacteriol. 140:251-260.

62. Lee, J. H., P. Patel, P. Sankar, and K. T. Shanmugam. 1985. Isolation and characterization of mutant strains of Escherichia coli altered in $H_2$ metabolism. J. Bacteriol. 162:344-352.

63. Lester, R. L., and J. A. DeMoss. 1971. Effects of molybdate and selenite on formate and nitrate metabolism in Escherichia coli. J. Bacteriol. 105:1006-1014.

64. Lim, S. T. 1978. Determination of hydrogenase in free-living cultures of Rhizobium japonicum and energy efficiency of soybean nodules. Plant Physiol. 62:609-611.

65. Ljungdahl, L. G. 1976. Tungsten, a biologically active metal. Trends in Biochem. Sci. 1:63-65.

66. Ljungdahl, L. G. 1980. Formate dehydrogenases: Role of molybdenum, tungsten and selenium, p.463-485. In M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.

67. Low, B. 1972. Escherichia coli K-12 F-prime factors, old and new. Bacteriol. Rev. 36:587-607.

68. MacGregor, C. H., and C. A. Schanitman. 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutant of Escherichia coli. J. Bacteriol. 114:1164-1176.

69. Macy, J., H. Kulla, and G. Gottschalk. 1976. $H_2$-dependent anaerobic growth of Escherichia coli on malate: succinate formation. J. Bacteriol. 125:423-428.

70. Mandel, M. and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53:159-162.

71. Mandrand-Berthelot, M., M. Y. K. Wee, and B. A. Haddock. 1978. An improved method for the identification and characterization of mutants of Escherichia coli deficient in formate dehydrogenase activity. FEMS Microbiol. Lett. 4:37-40.

72. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

73. Miller, J. B., D. J. Scott, and N. K. Amy. 1987. Molybdenum-sensitive transcriptional regulation of the chlD locus of Escherichia coli. J. Bacteriol. 169:1853-1860.

74. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

75. Murgola, E. J., and C. Yanofsky. 1974. Structural interactions between amino acid residues at positions 22 and 211 in the tryptophan synthetase alpha chain of Escherichia coli. J. Bacteriol. 117:444-448.

76. Pakes, W. W. C., and W.H. Jollyman. 1901. The bacterial decomposition of formic acid into carbon dioxide and hydrogen. J. Chem. Soc. 79:386-391.

77. Pardee, A. B., L. S. Prestidge, M. B. Whipple, and J. Breyfuss. 1966. A binding site for sulfate and its relation to sulfate transport into Salmonella typhimurium. J. Biol. Chem. 241:3962-3969.

78. Pascal, M. -C., J. -F. Burini, J. Ratouchniak, and M. Chippaux. 1982. Regulation of the nitrate reductase operon: effects of mutations in chlA, B, D and E genes. Mol. Gen. Genet. 188:103-106.

79. Pascal, M. -C., F. Casse, M. Chippaux, and M. Lepelletier. 1975. Genetic analysis of mutants of Escherichia coli K-12 and Salmonella typhimurium LT2 deficient in hydrogenase activity. Mol. Gen. Genet. 141:173-179.

80. Pascal, M. -C., and M. Chippaux. 1982. Involvement of a gene of the chlE locus in the regulation of the nitrate reductase operon. Mol. Gen. Genet. 185:334-338.

81. Patel, P. S. 1985. Biochemical genetics of hydrogen metabolism in Escherichia coli: purification and characterization of hydrogenase. Ph.D. dissertation, University of Florida.

82. Patrick, J. M., and W. J. Dobrogosz. 1973. The effect of cyclic AMP on anaerobic growth of Escherichia coli. Biochem. Biophys. Res. Comm. 54:555-561.

83. Pecher, A., F. Zinoni, and A. Bock. 1985. The seleno-polypeptide of formic dehydrogenase (formate hydrogen-lyase linked) from Escherichia coli: genetic analysis. Arch. Microbiol. 141:359-363.

84. Pecher, A., F. Zinoni, C. Jatisatienr, R. Wirth, H. Hennecke, and A. Bock. 1983. On the redox control synthesis of anaerobically induced enzymes in Enterobacteriaceae. Arch. Microbiol. 136:131-136.

85. Peck, H. D. Jr., and H. Gest. 1957. Formic dehydrogenase and the hydrogenlyase enzyme complex in coli-aerogenes bacteria. J. Bacteriol. 73:706-721.

86. Pinsent J. 1954. The need for selenite and molybdate in the formation of formic dehydrogenase by members of the Coli-aerogenes group of bacteria. Biochem. J. 57:10-16.

87. Ruiz-Herrera, J., A. Alvarez, and I. Figuera. 1972. Solubilization and properties of formate dehydrogenases from the membrane of Escherichia coli. Biochim. Biophys. Acta 289:254-261.

88. Ruiz-Herrera, J., and J. A. DeMoss. 1969. Nitrate reductase complex of Escherichia coli K-12: participation of specific formate dehydrogenase and cytochrome $b_1$ components in nitrate reduction. J. Bacteriol. 99:720-729.

89. Sankar, P. 1987. Biochemical genetics of $H_2$ metabolism: hydB, hydF and fhlA genes, Ph.D. dissertation, University of Florida.

90. Sankar, P., J. H. Lee, and K. T. Shanmugam. 1985. Cloning of hydrogenase genes and fine structure analysis of an operon essential for $H_2$ metabolism in Escherichia coli. J. Bacteriol. 162:353-360.

91. Sawer, R. G., S. P. Ballantine, and D. H. Boxer. 1985. Differential expression of hydrogenase isoenzymes in Escherichia coli K-12: evidence for a third isoenzyme. J. Bacteriol. 164:1324-1331.

92. Sawer, R. G., and D. H. Boxer. 1986. Purification and properties of membrane-bound hydrogenase isoenzyme 1 from anaerobically grown Escherichia coli. Eur. J. Biochem. 156:265-275.

93. Shah, P. A., R. R. Ugalde, J. Imperial, and W. J. Brill. 1984. Molybdenum in nitrogenase. Annu. Rev. Biochem. 53:231-257.

94. Shimokawa, O., and M. Ishimoto. 1979. Purification and some properties of inducible tertiary amine N-oxide reductase from Escherichia coli. J. Biochem. 86:1709-1717.

95. Showe, M. K., and J. A. DeMoss. 1968. Localization and regulation of synthesis of nitrate reductase in Escherichia coli. J. Bacteriol. 95:1305-1313.

96. Silhavy, T. J., and J. R. Beckwith. 1985. Uses of lac fusions for the study of biological problems. Microbiol. Rev. 49:398-418.

97. Silhavy, T. J., M. L. Berman and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

98. Silven, S. 1978. Transport of cations and anions, P. 221-324. In. B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.

99. Spencer, M. E., and J. R. Guest. 1973. Isolation and properties of fumarate reductase mutants of Escherichia coli. J. Bacteriol. 114:563-570.

100. Sperl, G. T., and J. A. DeMoss. 1975. chlD gene function in molybdate activation of nitrate reductase. J. Bacteriol. 122:1230-1238.

101. Stewart, V., and C. H. MacGregor. 1982. Nitrate reductase in Escherichia coli K-12: involvement of chlC, chlE, and chlG loci. J. Bacteriol. 151:788-799.

102. Takagi, M., T. Tsuchiya, and M. Ishimoto. 1981. Proton translocation coupled to trimethylamine N-oxide reduction in anaerobically grown Escherichia coli. J. Bacteriol. 148:762-768.

103. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of Escherichia coli. Bacteriol. Rev. 31:332-353.

104. Varenne, S., F. Casse, M. Chippaux, and M. -C. Pascal. 1975. A mutant of Escherichia coli deficient in pyruvate formate lyase. Mol. Gen. Genet. 141:181-184.

105. Waugh, R., and D. H. Boxer. 1986. Pleiotropic hydrogenase mutants of Escherichia coli K12: growth in the presence of nickel can restore hydrogenase activity. Biochimie 68:157-166.

106. Wimpenny, J. W. T., and J. A. Cole. 1967. The regulation of metabolism in facultative bacteria. III. The effect of nitrate. Biochim. Biochys. Acta 148:233-243.

107. Wimpenny, J. W. T., and D. K. Necklen. 1971. The redox environment and microbial physiology. I. The transition from anaerobiosis to aerobiosis in continuous cultures of facultative anaerobes. Biochim. Biophys. Acta 253: 352-359.

108. Wu, L. F., and M. -A. Mandrand-Gerthelot. 1986. Genetic and physiological characterization of new Escherichia coli mutants impaired in hydrogenase activity. Biochimie 68:167-179.

109. Yamamoto, I., and I. Ishimoto. 1978. Hydrogen-dependent growth of Escherichia coli in anaerobic respiration and the presence of hydrogenases with different functions. J. Biochem. 84:673-679.

110. Yerkes, J. H., L. P. Casson, A. K. Honkanen, and G. C. Walker. 1984. Anaerobiosis induces expression of ant, a new Escherichia coli locus with a role in anaerobic electron transport. J. Bacteriol. 158:180-186.

111. Zinoni, F., A. Beier, A. Pecher, R. Wirth, and A. Bock. 1984. Regulation of the synthesis of hydrogenase (formate hydrogen-lyase) of Escherichia coli. Arch. Microbiol. 139:299-304.

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